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Localization and quantification of leptin and leptin receptor in the canine corpus luteum, placenta and uterus during pregnancy

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2. Summary – Zusammenfassung

Leptin (Ob) was originally thought to be secreted exclusively by fat tissue regulating energy metabolism and satiety. Nowadays, it is known to be produced by many organs and to influence reproductive processes as well as other bodily functions. In the dog, little is known about Ob's role in reproduction. To investigate the potential involvement of Ob in canine pregnancy, we determined the localization of Ob and its receptor (ObR) by in situ hybridization (ISH) and immunohistochemistry (IHC) in the corpus luteum (CL), uterus and placenta of pregnant bitches and followed the changes in their gene expression levels by RT-PCR. Tissues were obtained from bitches ovariohysterectomized during pre- and post-implantation, mid-gestation and prepartum luteolysis. Also, uteri were collected from early pregnant dogs and their matched non-pregnant controls in diestrus. Ob and ObR mRNA and protein were detected during all stages of pregnancy in various cell types of the CL, uterus and placenta. ObR mRNA expression was increased in the pre-implantation uterus and in inter-placental sections at post-implantation. At placentation sites Ob mRNA was highest during post-implantation and prepartum luteolysis, and ObR expression was up-regulated prepartum. In the non-pregnant diestrus uterus Ob mRNA was below the detection limit, and ObR was not different between the groups. In conclusion, Ob may be involved in the establishment and maintenance of canine pregnancy in a paracrine/autocrine manner.

Keywords: leptin, corpus luteum, uterus, placenta, dog

Leptin (Ob) wird nicht nur vom Fettgewebe, sondern auch von vielen anderen Organen produziert und beeinflusst neben dem Energiestoffwechsel u.a. die Reproduktion. Zur Charakterisierung der Rolle von Ob während der kaninen Trächtigkeit wurde Ob und sein Rezeptor (ObR) mittels In-situ-Hybridisierung (ISH) und Immunhistochemie (IHC) in Corpus luteum (CL), Uterus und Plazenta von trächtigen Hunden lokalisiert und ihre Genexpression mittels RT-PCR bestimmt. Das Gewebe stammt von Hunden, die in verschiedenen Trächtigkeitsphasen (prä-Implantation, post-Implantation, Mitte Trächtigkeit, präpartale Luteolyse) ovariohysterektomiert wurden. Zusätzlich wurden Uteri von früh-trächtigen Hunden und nicht-trächtigen Kontrolltieren im Diöstrus untersucht. Sowohl Ob und ObR mRNA als auch Protein wurden in den untersuchten Trächtigkeitsphasen in verschiedenen Zelltypen von CL, Uterus und Plazenta nachgewiesen. Die ObR mRNA Expression war im prä-Implantationsuterus sowie in inter-plazentären Abschnitten während der post-Implantationsphase erhöht. Im Bereich der Plazentationsstellen wurden die höchsten Ob mRNA-Werte während post-Implantation und präpartaler Luteolyse gemessen, während ObR mRNA präpartal hochreguliert war. In den nicht-trächtigen Diöstrus-Uteri war keine Ob mRNA nachweisbar und die ObR mRNA unterschied sich zwischen den Gruppen nicht. Daher scheint Ob parakrin/autokrin an der Etablierung und Aufrechterhaltung der kaninen Trächtigkeit beteiligt zu sein.

Schlüsselwörter: Leptin, Corpus luteum, Uterus, Plazenta, Hund

3. Abstract

Leptin (Ob) was originally thought to be secreted exclusively by the adipose tissue regulating energy metabolism and satiety [1]. Nowadays it is known to be produced by many other organs and to influence immune response, angiogenesis, haematopoiesis and reproductive processes, as well. Ob and its receptor (ObR) were found in the corpus luteum (CL), uterus and placenta of several species both at the mRNA and protein level. Potential roles for Ob in luteal steroidogenesis, embryo-maternal communication, trophoblast cell invasion and placental and fetal growth have been proposed [2-5]. In the dog, the link between peripheral Ob levels and obesity, nutritional and metabolic status has already been established however, little is known about its role in canine reproduction. Luteal Ob was detected by immunohistochemistry (IHC) in non-pregnant bitches, but ObR was not found and specific cellular localization was not described [6]. Recently, gene expression of Ob and its receptor was determined by our group using semi-quantitative real time PCR (RT-PCR) in the canine CL [7], but their specific localization was not examined. Furthermore, there is no information whether Ob signalling within the uterus plays a role in the establishment and maintenance of pregnancy in the dog.

As a preliminary step for Ob's and its cognate receptor's functional characterization, we determined the localization of Ob and ObR at the mRNA and protein level by in situ hybridization (ISH) and IHC, respectively, in the CL, uterus and placenta of pregnant bitches. To investigate changes in gene expression levels of Ob and ObR over the course of pregnancy, semi-quantitative RT-PCR was performed. In addition, to evaluate whether leptin signalling is involved in embryo-maternal communication in the uterus during early pregnancy, uterine Ob and ObR mRNA concentration was compared between non-pregnant dogs in early diestrus (E-) and dogs in the pre-implantation period (E+). CL, utero-placental and inter-placental (including the pre-implantation stage) uterine sections were obtained from healthy bitches (2-8 years, different breeds) which were mated 2 days after ovulation and ovariohysterectomized at pre-implantation (days 8-12 after mating), post-implantation (days 18-25), mid-gestation (days 35-40) and at prepartum luteolysis. Dogs in early diestrus were matched to the pre-implantation group based on ovulation timing.

Ob and ObR mRNA and protein were detected during all stages of pregnancy in various cell types of the canine CL, uterus and placenta. In the pre-implantation uterus and inter-placental uterine sections, Ob mRNA levels did not change significantly, whereas ObR expression was increased during pre- and post-implantation compared to mid-gestation. In the utero-placental uterine sections, Ob mRNA expression was significantly up-regulated during post-implantation and prepartum luteolysis compared to mid-gestation. Placental ObR mRNA concentration was higher at prepartum luteolysis than at post-implantation and mid-gestation. Ob expression was below the detection limit in the E- group and there was no difference in ObR mRNA levels between the E+ and E- groups.

We conclude that Ob and its receptor may be involved in the establishment and maintenance of canine pregnancy in a paracrine/autocrine manner. Our results suggest a regulatory role for the Ob-ObR system in luteal function, endometrial receptivity, implantation, trophoblast invasion, endothelial function and angiogenesis in reproductive tissues. More studies are needed to create a discrete basis of knowledge on how exactly and to which degree Ob might influence reproductive processes in the dog.

4. Introduction: Literature review

4.1. The canine reproductive cycle

The canine female reproductive cycle is controlled by the hypothalamic-pituitary-ovarian axis. Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus in a pulsatile manner triggering the gonadotroph cells of the anterior lobe of the pituitary gland to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the same pulsatile fashion [8]. In response to these gonadotropins, the granulosa, theca, and luteal cells of the ovary secrete the sexual steroid hormones estradiol and progesterone. Both the ovarian hormones and the gonadotropins exert a feedback to the superior hormone producing centres of the hypothalamic-pituitary axis [8].

The estrous cycle of the domestic dog (Canis familiaris) is defined as monoestrous and non-seasonal with spontaneous ovulation [9]. It includes 4 stages that were first described in 1900 by Heape [10]: proestrus, estrus, diestrus and anestrus. The onset of the first proestrus indicates puberty, which occurs between 6 and 24 months with small breeds usually having earlier puberty than large ones [11].

The first stage of the cycle, *proestrus*, has an average duration of 9 days ranging normally from 1 to 27 days [12] and is characterized by vulvar swelling, serosanguineous vaginal discharge and attractiveness to males. This is the phase in which serum concentrations of estradiol peak triggering the preovulatory surge of LH.

Estrus is the stage following proestrus and is defined by the bitch's acceptance of the male for mating. Its duration ranges from 4 to 24 days with an average of 9 days [12]. The vulva is still enlarged but typically less turgid than in proestrus and the vulvar discharge contains less blood and thus is becoming more "straw-colored". The preovulatory LH surge triggers ovulation, which occurs approximately 2 days later [13].

Preovulatory luteinization of follicles already starts in estrus simultaneously with the LH peak and continues after ovulation leading to formation of corpora lutea. A gradual increase of peripheral progesterone from basal levels (<1.0 ng/ml) up to a peak of 15 to 90 ng/ml is detected [14]. This third stage of the canine reproductive cycle that follows ovulation is called *diestrus, metestrus* or *the luteal phase*. While the external signs of heat regress and finally stop, this phase is characterized by the presence of corpora lutea, which are the only

source of progesterone in the bitch [15], and progesterone dominance. The luteal phase lasts 8 to 12 weeks in non-pregnant dogs while the CL slowly regresses. In pregnant bitches, an abrupt fall of progesterone due to the release of placental prostaglandins occurs approximately 65 days after the preovulatory LH peak [16, 17].

Anestrus starts once serum progesterone reaches levels below 1.0 ng/ml. The duration of this phase is highly variable ranging from 3-10 months [15]. During this time, the uterus undergoes self-repair and complete involution.

4.2. The canine pregnancy

Normal gestation length calculated from the first day of mating until birth ranges from 57 to 72 days with an average of 63 days. If the time of the LH peak or ovulation is known, parturition date can be assessed more accurately: normal duration of pregnancy is 64 to 66 days if calculated from the preovulatory surge in LH (which is simultaneous with the initial rise of progesterone), or 62-64 days if calculated from ovulation, since ovulation has been estimated to happen 2 days after the LH peak [18, 19].

Unlike in most species, canine oocytes are immature at ovulation arrested in the germinal vesicle stage of the first meiotic division and must undergo further maturation steps until reaching Metaphase II stage 2-3 days later, when they can be fertilized by spermatozoa [19, 20]. Oocytes remain viable for an additional 3-5 days after maturation [21]. Fertilization and early embryonic development takes place within the oviducts before the embryo enters the uterus approximately 11 days after the preovulatory LH peak as a morula/early blastocyst [20, 22-24]. After migration of the blastocysts between both cornua during days 12-17, final situating of embryos occurs on days 16-18 with actual attachment and trophectoderm invasion to the endometrium on day 22 after the LH peak [23, 25-27]. At the same time, the placenta starts to develop.

With the implantation of the blastocyst, the period of the embryo begins, and after completion of organogenesis, the fetal period starts [28]. This last part of development (from ~day 35 of gestation) is characterized by rapid growth and the appearance of external features like pigmentation, hair and claws and sexual differentiation [28].

4.3. The canine corpus luteum: anatomy and histology

The ovary is divided into an inner zona vasculosa and an outer zona parenchymatosa. The corpora lutea, which reach a size of 3-12 mm in diameter at peak growth [29] as well as the follicles are located within the zona parenchymatosa. In contrast, the zona vasculosa mainly consists of vessels and connective tissue.

In the bitch, the only source of progesterone during pregnancy as well as in the non-pregnant luteal phase is the CL [14]. Before ovulation, follicular vessels become congestive and the wall of the follicle thickens. In the dog, the ovulation process is subtle and not accompanied with extensive haemorrhage. The site of rupture appears as a small red area (stigma), and collapse of the follicle is not consistently observed [30-32]. After ovulation, the CL becomes further vascularized by an extensive capillary network [33]. These capillaries originate from the theca interna and penetrate the basement membrane into the luteinized granulosa cell layer [29]. Under the influence of LH, luteinization occurs, which means the rapid transformation of granulosa and theca cells into luteal cells [15]. This transformation includes hypertrophy and hyperplasia [33] as well as the storage of carotinoids resulting in an increase in size and giving the CL a yellowish appearance. In the dog, there is only one type of luteal cell that resembles the large luteal cells of ruminants, and there are considerable differences in cell size [15, 34] (Figure 1).

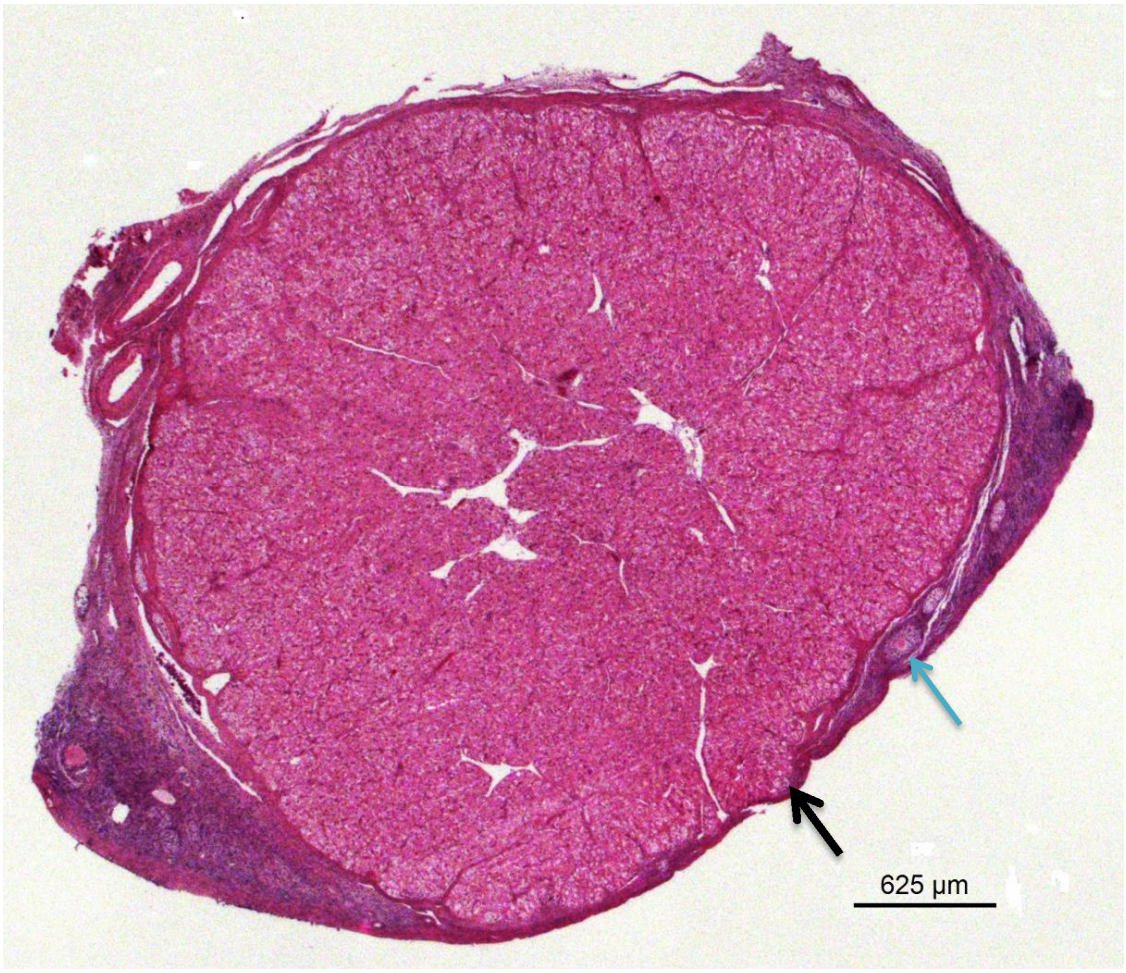


Figure 1: Cross-section of a canine ovary with a large corpus luteum (black arrow) and a small follicle (blue arrow); HE staining

4.4. The canine uterus: anatomy and histology

The uterus is divided into a short body (corpus uteri) and 2 long horns (cornua uteri), and is separated from the vagina by the cervix. The uterine wall is composed of 3 layers: *endometrium*, *myometrium* and *perimetrium* [33].

The *endometrium* consists of the luminal lamina epithelialis and the underlying lamina propria. The cells of the lamina epithelialis are simple cuboidal or columnar [35] depending on the stage of the cycle. The lamina propria is characterized by the presence of superficial and deep uterine glands embedded in connective tissue.

The *myometrium* is divided into an inner circular layer (stratum circulare) and an outer longitudinal layer (stratum longitudinale). These two muscle layers are separated by a richly vascularized and well-innervated stratum vasculare [33, 36].

The endometrium and myometrium undergo cyclic changes of growth and differentiation under the influence of steroid hormones corresponding to the reproductive stage of the bitch [29, 37].

The *perimetrium* or tunica serosa represents the outermost layer of the uterus consisting of loose connective tissue covered by a simple squamous mesothelium [33, 36]. Numerous blood and lymphatic vessels as well as nerve fibres are present in this layer [33] (Figure 2).

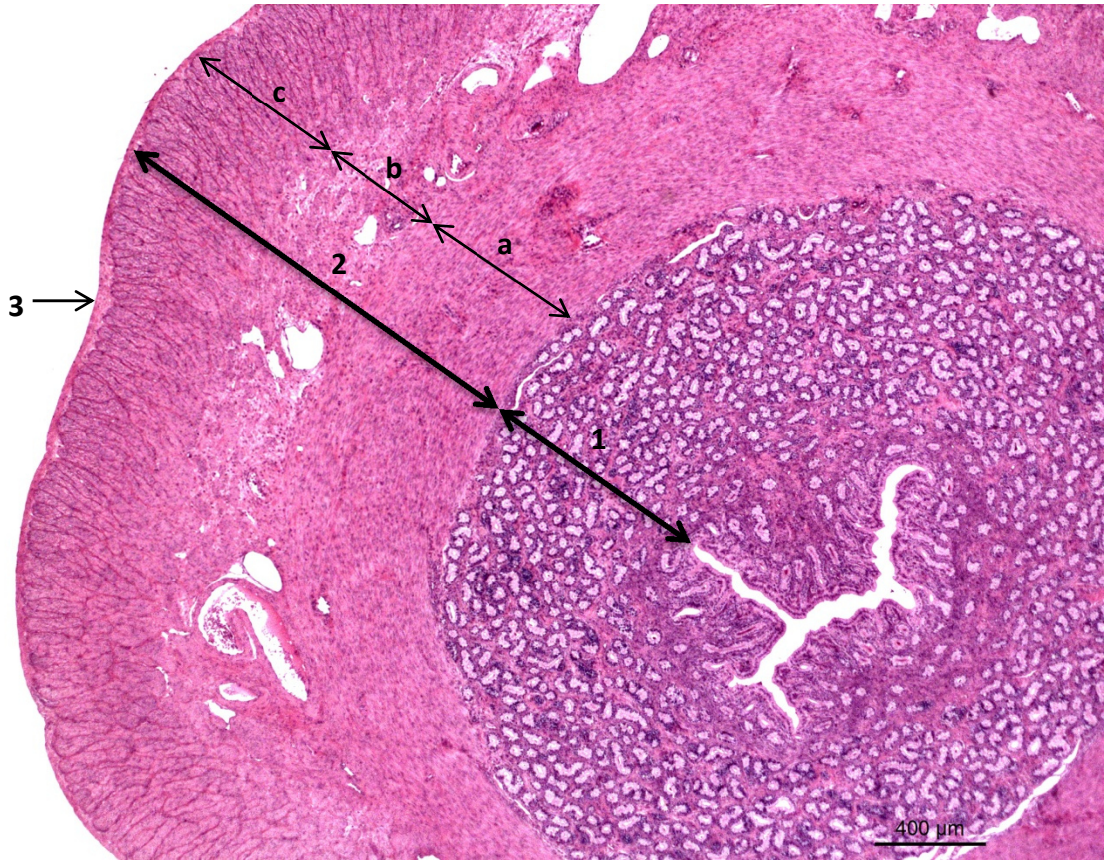


Figure 2: Cross-section of the canine uterine horn; HE staining

1) Endometrium
2) Myometrium
    a. Stratum circulare
    b. Stratum vasculare
    c. Stratum longitudinale
3) Perimetrium

4.5. The canine placenta: anatomy and histology

The canine placenta is called *zonary*, because the chorionic villi are distributed in the form of an encircling girdle [38] (Figure 3a). By the form of the feto-maternal interdigitation, the dog placenta is called *lamellar* [39]. Another classification scheme is based upon the degree of proximity of maternal and fetal blood circulations. In the bitch, fetal trophoblasts invade the endometrium under the loss of endometrial epithelium and connective tissue so that they are directly exposed to the maternal capillaries [40]. Thus, the canine placenta is called *endotheliochorial*, implicating a tight adherence between fetal and maternal parts. The third term used for the canine placenta is *deciduate*, because with the expulsion of the fetal membranes and the placenta at parturition, a portion of the endometrium is shed as well. This shed part of the endometrium is called decidua [40]. The resulting lesions within the endometrium will be repaired in the subsequent puerperium. Additionally, in respect to the geometric arrangement of fetal and maternal capillaries and blood flows within the placental

labyrinth, the carnivores' exchange system can be classified as *simple crosscurrent* with blood flow directions being mainly vertical to each other [41].

At the edges of the placental girdle some of the maternal endothelium is destroyed by trophoblasts leading to local bleeding and so-called marginal haematomas between the paraplacenta and the chorionic girdle [38, 40, 42]. The erythrocytes within these vascular lakes are phagocytosed by specialized phagocytic/absorptive cytotrophoblasts and are thought to serve as a source of iron for the embryo [40, 42]. The breakdown of haemoglobin into uteroverdin results in a green colour of the marginal haematomas typical for the canine placenta [23, 40].

The chorionic villi of the embryonic trophectoderm protrude into the maternal endometrium reaching to the deep end of the glands with advanced pregnancy [33] and forming chorionic lamellae. In between these lamellae of fetal origin that are branched in the dog, endometrial lamellae develop [38], and together they form the placental labyrinth [38]. The chorionic epithelium becomes two-layered with the outer layer formed by fusion of many cytotrophoblast cells into multinuclear syncytiotrophoblasts [36], and the inner layer of the cytotrophoblasts continuously destroying the endometrial epithelium. Syncytiotrophoblasts tend to be more invasive than cytotrophoblasts [38]. The superficial endometrial glands are extended to glandular chambers which are particularly large in dogs [38, 43]. Between the deep glands and the glandular chambers, a cover layer of endometrial connective tissue is built separating the two layers of glands [38]. Above the glandular chambers, the placental labyrinth formed by chorionic and endometrial lamellae is found [38]. Here, the maternal tissue is destroyed up to the capillaries resulting in an intimate contact between maternal endothelium and chorionic epithelium [38]. The increasing density of blood vessels in the lamellar region as pregnancy progresses, assures nutrient transfer to the growing fetus. Decidual cells derived from maternal fibroblasts are also found within the placental lamellae between maternal capillaries and trophoblast cell [44]. In addition, fetal capillaries, which are smaller and thinner-walled than maternal capillaries, are spread within the placental labyrinth [33] (Figure 3b).

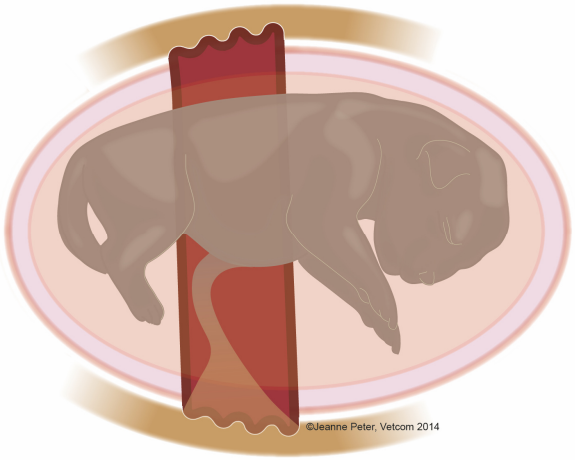


Figure 3a: girdle-shaped placenta of a canine fetus at the end of pregnancy

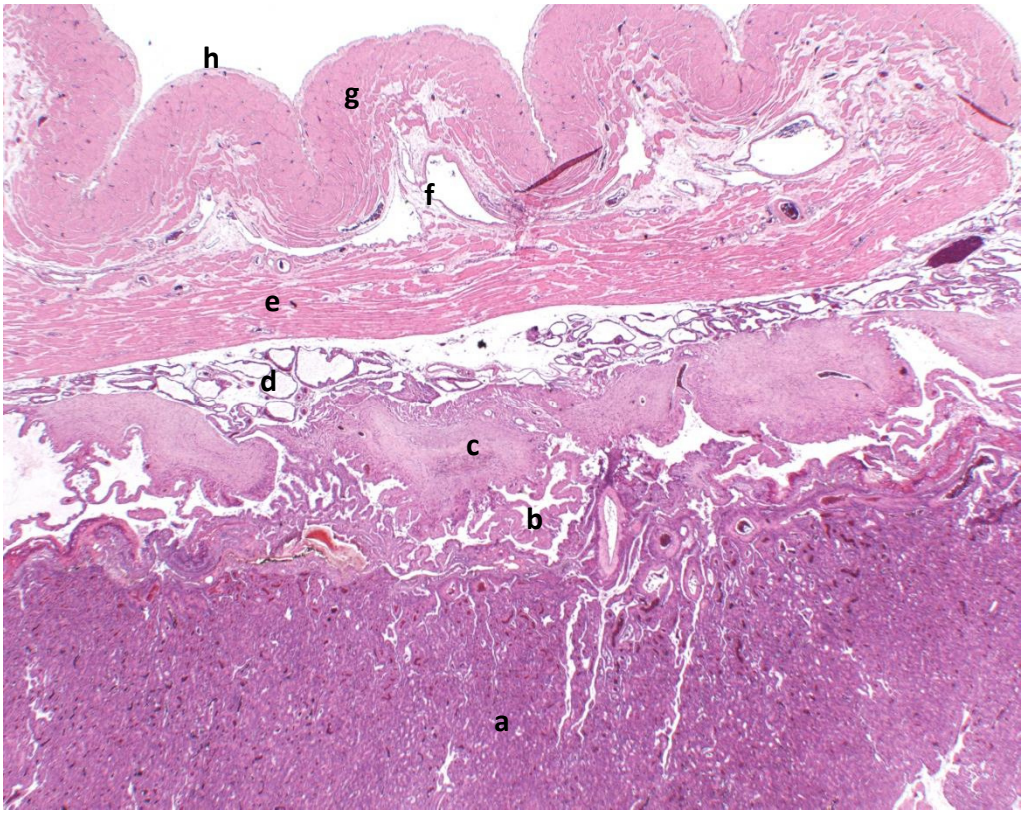


Figure 3b: Canine utero-placental section (during prepartum luteolysis); HE staining
a) Placental labyrinth
b) Glandular chambers
c) Cover layer of endometrial connective tissue
d) Deep uterine glands
e) Stratum circulare of the myometrium
f) Stratum vasculare of the myometrium
g) Stratum longitudinale of the myometrium
h) Perimetrium

4.6. Leptin and leptin receptor

This section is structured as follows: first, literature is reviewed from a wide range of species, and in the second part studies focusing on leptin in the dog are summarized.

Leptin (Ob) derives from the Greek word “leptos” meaning “thin”. Leptin is the product of the obese (ob) gene and was first discovered in mice by Zhang and his group in 1994 using positional cloning strategies [45]. It is a protein of 167 amino acids [45], has a molecular weight of 16 kDa and is structurally similar to the type I cytokine superfamily [46]. Originally, Ob was thought to be secreted exclusively by the adipose tissue [1], but nowadays it is known to be produced by other organs e.g. the stomach [47, 48], skeletal muscle [49], pancreas [50], uterus [51, 52], placenta [53, 54], pituitary gland [55, 56] as well as the mammary gland [57, 58] of several species.

The leptin receptor (ObR) was first identified in the mouse by Tartaglia and his group in 1995 by expression cloning techniques [59] and is structurally similar to class I cytokine receptors [60]. Due to alternative splicing of a single leptin receptor gene, several isoforms of ObR

have been described in humans and rodents (ObR-a, ObR-b, ObR-c, ObR-d, ObR-f) differing in the length of their intracellular domain. Additionally, there is a soluble isoform (ObR-e) lacking of intracellular and transmembrane parts and thus consisting only of an extracellular domain [60, 61]. Leptin's effect on feed intake is mediated by hypothalamic ObR-b (long isoform) [61, 62], while the short isoforms may serve as transporters through physiological barriers (e.g. blood-brain-barrier [63]), and control Ob internalization and degradation within cells [64]. The soluble isoform may act as a leptin-binding protein controlling the bioavailability of free Ob [65, 66]. Also, clearance of leptin from the circulation is delayed if it is bound to ObR-e potentiating Ob's effect [67]. ObR-b is present in abundance in the hypothalamus, but both long and short isoforms of ObR were detected in peripheral organs e.g. the liver, lung, heart, kidney, small intestine, adipose tissue, spleen, adrenal glands, and in the fetus and placenta explaining leptin's pleiotropic actions [61, 68-71].

Besides Ob's well-known role in regulating food intake and energy metabolism, emerging evidence suggests that Ob influences many physiological processes e.g. immune response, haematopoiesis, endothelial function, angiogenesis and reproduction. Most of our knowledge about Ob's physiology has come from studies using rodents with mutations of the Ob or ObR gene, the so called *ob/ob* or *db/db* mice, respectively (see below).

4.6.1. Leptin in non-reproductive processes

4.6.1.1. Regulation of food intake and energy metabolism

Leptin controls satiety, food intake and energy metabolism by informing the central nervous system (CNS) about the size of fat stores and feeding status. More specifically, it decreases food intake and increases energy expenditure (Figure 4a).

It has been shown in several species including the dog that there is a positive correlation between plasma Ob concentrations and body fat content [72-75]. High serum levels of Ob signal to the brain the presence of sufficient energy stores and thus result in reduced appetite and increased energy expenditure [76]. On the other hand, food restriction leads to a decrease in serum Ob levels as a physiologic response to starvation [77].

The *ob/ob* mice [78] are characterized by a defective circulating Ob protein due to an inactivating gene mutation. These mice suffer from obesity, hyperphagia, hyperglycemia, insulin resistance, reduced energy expenditure and infertility. All these symptoms are reversible when the recombinant Ob protein is administered [76, 79, 80]. In contrast, in *db/db* mice [81, 82] (Figure 4b), which are characterized by a mutation in the ObR-b gene and thus lack the functional long ObR isoform, injection of Ob does not result in re-establishment of fertility and normalization of food intake and body weight [76]. Additionally, when Ob was administered to wild type mice, a dose-dependent loss of body weight was observed [83].

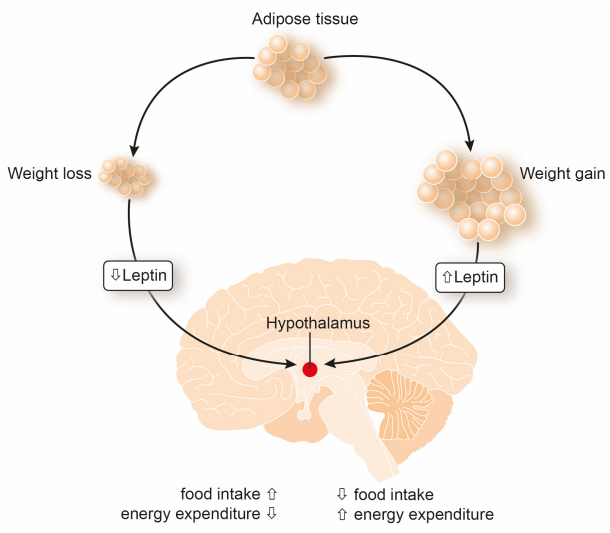


Figure 4a: Circulating leptin, which is proportional to the amount of fat stores, informs the CNS about the state of energy balance in order to adjust food intake and energy expenditure. © Jeanne Peter, Vetcom 2014, adapted from Ricci et al. [84].

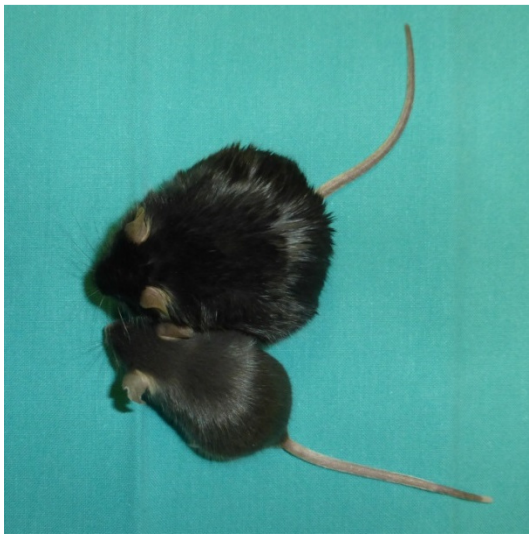


Figure 4b: The *db/db* mouse (above) is extremely obese as compared to the control animal below; by curtesy, T. Lutz and CN. Boyle, Institute of Veterinary Physiology, University of Zurich.

4.6.1.2. Immune response

It is known that malnutrition predisposes to death from infectious diseases [85]. Of course many factors are involved in this predisposition and Ob is supposed to be one of them. Low body fat content and thus Ob deficiency increases susceptibility to infectious stimuli [86], but also obesity-associated hyperleptinemia is known to impair immune response [87]. A regulating role of Ob both in innate and adaptive immunity has been demonstrated in several *in vivo* and *in vitro* studies using mice lacking functional Ob (*ob/ob)* or ObR (*db/db)* and thus showing inadequate immune response [88].

Ob levels increase during infection and inflammation, and Ob might be part of the mechanisms causing anorexia during acute inflammatory conditions [89, 90].

Loffreda and his group showed in *ob/ob* and *db/db* rodents that Ob activates macrophages and induces their expression of proinflammatory cytokines [91]. Furthermore, Ob is thought to modulate neutrophil activation, chemotaxis and phagocytosis as well as to have an influence on natural killer cell maturation, differentiation, activation and cytotoxicity [92].

Beside deficiency of the *ob/ob* and *db/db* rodents concerning the innate immunity, these animals also show deficits in respect to the adaptive/acquired immune response. In a study of Howard et al. *ob/ob* mice showed marked reduction in size and cellularity of the thymus which could be reversed by peripheral administration of recombinant Ob. They also demonstrated *in vitro* that Ob protected thymocytes from steroid-induced apoptosis [93]. In addition, Lord and his colleagues found expression of ObR by T lymphocytes suggesting that Ob modulates directly the T cell mediated immune response [94].

Moreover, the sequence homology of ObR to members of the class I cytokine superfamily as well as the detection of ObR expression in several immune cells like e.g. neutrophils, natural killer cells, lymphocytes and monocytes may also indicate a role for Ob in the regulation of the immune response [92].

Last but not least, Ob influences immune response also by modulating haematopoiesis [95] (see below).

4.6.1.3. Haematopoiesis

The structural similarity of Ob and its receptor to molecules involved in the regulation of haematopoiesis, and the detection of ObR expression on a diverse range of haematopoietic cells gave rise to studies exploring the potential role of Ob in the modulation of haematopoiesis [96].

The bone marrow, which is the major site of haematopoiesis in adult mammals, contains a heterogeneous network of cells including endothelial cells, fibroblasts, adipocytes, macrophages, and both mature and immature haematopoietic cells. Bennet et al. [95] detected ObR mRNA in both human and murine haematopoietic stem cell populations as well as Ob expression in haematopoietic stroma. In vitro, Ob increased the formation of myeloid, lymphoid and erythroid colonies in murine fetal liver stem cells, while lympho- and myeloproliferative capacity of the *db/db* and *ob/ob* mice was compromised [95]. Mice kept on a high fat diet showed higher serum Ob levels and significantly increased bone marrow nucleated cell numbers, particularly lymphocytes, compared to lean controls [97] further suggesting that Ob signalling is necessary for adequate haematopoiesis.

Gainsford and Alexander [96] reviewed Ob's role in haematopoiesis and concluded that although Ob has proven active in a number of *in vitro* assays on haematopoietic proliferation, it mainly acts as a synergistic factor enhancing the effects of other regulators. They also mention the potential indirect influence of Ob through actions upon the haematopoietic microenvironment.

4.6.1.4 Endothelial function and angiogenesis

Further roles for leptin have been postulated in the circulatory system as ObR expression was found on endothelial cells suggesting that the endothelium may be a target for leptin's actions [98, 99].

Sierra-Honigmann et al. [98] demonstrated both *in vitro* and *in vivo* angiogenic activity of leptin. First, ObR was shown to be expressed and functional on human umbilical vein endothelial cells *in vitro* and in a further step, *in vivo*, Ob led to neovascularization in normal rats' corneas but not in corneas of *db/db* rats. The proliferative influence of Ob on endothelial cells has also been reported by Bouloumie et al. [100], and also Anagnostoulis et

al. confirmed a significant angiogenic effect [101]. In this latter study, discs containing different doses of leptin were implanted subcutaneously in rats, removed after 7 and 14 days and examined morphologically and by IHC. Leptin had an angiogenic effect which was dose- and time-dependent and equivalent to the angiogenic effect of vascular endothelial growth factor (VEGF).

VEGF is an important angiogenic factor that controls endothelial cell proliferation and survival [102]. Ob regulates VEGF expression as it has been shown in murine ovaries [103]. Cao et al. implanted pellets containing Ob and/or VGEF into mouse corneas and found that leptin promoted angiogenesis alone and - much more intensely - synergistically with VEGF [104].

Besides stimulating angiogenesis via VEGF, Ob might also exert its effects through extracellular matrix remodelling by regulating the expression of matrix metalloproteinases (MMPs) [5]. This is important for capillary formation, because pre-existing blood vessels need to locally degrade the underlying basal lamina and invade the stroma so that vascularization can be accomplished. MMPs are crucial factors involved in this process [105, 106].

Furthermore Ob has also been shown to cause *vasodilation*, although findings are inconsistent about its mechanism of action. Kimura and coworkers state that Ob's vasorelaxing effect is mediated by endothelium-derived nitric oxid (NO) [107], whereas other groups show a NO-independent vasodilation [108, 109].

In addition to the vasodilatory action of Ob, it also exerts a *hypertensive activity* through stimulation of the sympathetic nervous system [110, 111]. This is prominent in chronic hyperleptinemia, when the pressor effect is the predominant among all cardiovascular effects of Ob [112].

In humans, obesity is an important risk factor for cardiovascular disease, and the associated hyperleptinemia can result in *leptin resistance* [113], which is characterized by a failure of elevated circulating Ob to counteract obesity [114]. Mark et al. postulated the so-called "selective leptin resistance" theory explaining how high Ob levels can contribute to increasing sympathetic activity and arterial pressure in obese individuals who, at the same time, show resistance to the metabolic (satiety and weight-reducing) actions of Ob [112].

In adult tissue, angiogenesis normally occurs during tissue repair (e.g. in wound healing), or inappropriately associated with pathological conditions including tumour growth [115-117]. As the female reproductive organs (i.e. ovaries, uterus, placenta) are subject to periodic and extremely rapid growth and regression and thus to striking changes in blood flow, they are some of the few adult tissues where angiogenesis is normal [116, 117]. Processes depending at least partly on angiogenesis are e.g. regulation of follicular growth, atresia and selection of the dominant follicle destined to ovulate [102, 118-120], development of the CL [121] and support of fetal growth via placental vasculature [122].

4.6.2. Leptin in female reproduction

Leptin is regarded as an important link between energy/feeding status and reproductive function. It enables the hypothalamic-pituitary-gonadal axis to respond to changes in the animal's caloric status, and thus helps an animal during periods of metabolic stress to put energy rather in survival than in processes such as reproduction [123].

It has been shown that women have higher plasma leptin levels than men with similar fat mass [124, 125]. The reason for this sexual dimorphism in plasma Ob levels is still unclear. One possible explanation is the influence of sexual steroids with estrogens increasing Ob production [126] and androgens suppressing it [127]. Ovariectomy in rats reduced Ob production in adipose tissue and led to a decline in serum Ob levels and to significant weight gain. The administration of estradiol (E2) reversed these effects of ovariectomy [128]. The involvement of estradiol is also supported by the fact that serum Ob concentrations are lower in postmenopausal than in premenopausal women [126]. However, estrogen does not seem to be pivotal for this sexual dimorphism as postmenopausal women had still higher Ob levels than men of comparable age [126]. Differences in fat distribution (subcutaneous fat expresses more Ob than intraabdominal [129]) might be involved in the mechanism of sexual dimorphism. Additionally, female adipose tissue might be more sensitive to hormones stimulating Ob secretion (e.g. insulin), or females may be less sensitive to Ob's lipostatic actions leading to a compensatory increase in Ob production [125].

4.6.2.1. Onset of puberty

Puberty is defined by the attainment of sexual maturity. It is known from several species including humans that a threshold body fat content is necessary for the onset of puberty and normal reproductive function [130]. In obese girls puberty is observed at a younger age than in normal weight subjects [131, 132]. On the other hand, puberty is delayed and menstruation stops in extremely thin adolescent women. The administration of exogenous leptin accelerates sexual maturation in normal female rodents [133], and in humans with congenital Ob deficiency, it results in puberty reached at an appropriate age [134]. A possible interpretation of the correlation between onset of puberty and energy status could be as follows. Leptin levels signal to the hypothalamus that the energy stores are adequate to nourish a conceptus during a possible pregnancy [135]. Leptin may stimulate hypothalamic cells to release GnRH and thus trigger the resulting release of gonadotropins and the subsequent production of gonadal steroids. This hypothesis is supported by a study in which gonadotropin levels were shown to be low in *ob/ob* mice [136]. Whether Ob acts directly or indirectly on GnRH neurons there are controversial discussions (reviewed by Hill et al. [123] and Martos-Moreno et al. [137]). In contrast to a study where ObRs were found in immortalized gonadotropin-releasing hormone-secreting neurons suggesting a direct action [138], most authors deny the physiological expression of leptin receptors in hypothalamic GnRH producing neurons (reviewed by Hill et al. [123]). Instead - or additionally to the direct effect - Ob's stimulatory influence on GnRH secretion might be

mediated, at least partially, through kisspeptin producing neurons, which do express ObR [137]. Martos-Moreno et al. [137] mention that Ob decreases the hypothalamic expression of neuropeptide Y (NPY) and thus decreases the inhibitory effect of NPY on pulsatile GnRH secretion indirectly.

It is likely that leptin rather has a permissive role and is not the only factor involved in the initiation of puberty [139].

4.6.2.2. Fertility – Infertility

It is well-known that *ob/ob* mice are sterile. Correction of leptin deficiency by administration of recombinant leptin restores fertility indicating a permissive role for Ob [79, 140]. Abnormal reproductive function has also been reported in human patients with a missense mutation in the Ob gene [141]. In addition, interrupted reproductive cycles have often been observed in humans with low Ob levels e.g. anorectic individuals, elite athletes or ballet dancers [142]. In these cases of so-called functional hypothalamic amenorrhea, recombinant human Ob replacement improved reproductive function [142].

4.6.2.3. Ovarian function

Although Ob has generally been thought to influence reproduction principally at the neuroendocrine level, evidence has arisen to indicate its direct involvement in ovarian activity.

Both Ob and ObR mRNA and protein have been detected in the ovaries of several mammalian species [4, 7, 106, 143-149] suggesting an endocrine and/or autocrine/paracrine regulatory role.

There are numerous *in vitro* and *in vivo* studies about Ob's effect on ovarian steroidogenesis often yielding contradictory results. Most of these studies suggested that the effect of Ob on ovarian cells is *inhibitory* either directly [150] or indirectly by attenuating e.g. gonadotropin- or insulin like growth factor 1 (IGF-1)-mediated steroidogenesis [151]. Lin et al. reported a down-regulating effect of Ob on cAMP-stimulated steroidogenic acute regulatory (StAR) protein, the rate limiting de novo protein in progesterone synthesis [152]. There are contrasting studies revealing direct and indirect *stimulatory* effects of Ob on ovarian steroidogenesis [4, 153]. Nicklin et al. showed in bovine luteal cells *in vitro* that in the presence of IGF-1 (100 ng/ml), Ob increased progesterone production significantly, whereas Ob alone had no effect [4]. Additionally, Ruiz-Cortés and her group described a biphasic effect in porcine granulosa cells, where StAR and progesterone production were increased by low (10 ng/ml; physiological) and reduced by high (1000 ng/ml) dosages of Ob [154]. Similar results were obtained in human luteinized granulosa cells, where estradiol and

progesterone secretion was stimulated by low (1 and 10 ng/ml) and suppressed by high doses of Ob (100 ng/ml) [155].

These findings could at least partly explain the higher incidence of infertility in obese subjects usually having elevated plasma Ob levels.

Furthermore, Ob is hypothesized to influence maturation and selection of developing follicles [156] as well as CL formation, function and regression.

A study by Joo et al. [103] shows that treatment with Ob and gonadotropins during superovulation in aged mice resulted in an increased number and developmental competence of oocytes ovulated compared to the control group which was only treated with gonadotropins. In addition, ovarian VEGF expression was significantly increased in the Ob-treated groups compared to the control group. According to the authors the stimulatory effect of Ob may be mediated by increased expression of ovarian VEGF and may imply a potential clinical importance in the treatment of age-related reduced fertility.

Another mechanism by which Ob influences follicular development was described by Almog et al. [156] where Ob accelerated follicular maturation by attenuating apoptosis. In their study, recombinant leptin was injected daily to immature female rats. Besides reaching puberty earlier, treated animals showed in histology hypertrophy of granulosa cells, higher number of ovulations and a highly significant decrease in the incidence of follicular apoptosis compared to untreated controls.

Additionally, Ob seems to regulate ovarian function centrally through the control of gonadotropin secretion [157, 158]. Injection of leptin into the third ventricle of estrogen-primed ovariectomized adult female rats resulted in a highly significant increase in plasma LH [157]. According to Yu et al. [157], this effect was presumably caused by the release of GnRH; however, a direct effect on the LH secreting cells in the pituitary is also likely, as Ob's potency to release LH from incubated pituitaries *in vitro* is demonstrated in the same paper.

On the other hand, steroid hormones may also affect ovarian Ob production. Both estradiol and progesterone increased Ob secretion *in vitro* by porcine luteal cells collected during the mid-luteal phase of the estrous cycle [145].

4.6.2.4. Pregnancy

The role of Ob during pregnancy and fetal development differs considerably between species and is not well understood in any [159].

4.6.2.4.1. Hyperleptinemia and leptin resistance

Ob concentrations in maternal serum are significantly elevated during pregnancy and decrease around birth as shown in baboons [160], mice [161], humans [162], rats [163] and

mares [164]. Consequently a role for Ob in the maintenance of pregnancy and regulation of maternal energy balance is suggested.

Increased Ob levels in gestation seem to be a ubiquitous feature in mammals [165], however its *source* is controversially discussed and is probably species-specific. The increase in maternal fat stores does not seem to correlate with the changes in circulating Ob [53]. The significant increase in peripheral Ob concentration already in early pregnancy before any notable fat deposition implies sources other than the adipose tissue only [166]. Ob levels also drop significantly after parturition and remain low postpartum [53, 160, 167, 168]. One possible explanation for the elevated Ob levels during pregnancy is placental synthesis. Ob expression in the placenta was detected e.g. in humans and rodents [53, 54, 69, 169]. In contrast, Malik et al. did not detect any Ob expression in mouse placenta by RT-PCR [167]. Henson and his colleagues [160] conducted a study on pregnant baboons. While maternal Ob levels increased dramatically with gestational age, placental Ob mRNA decreased with advancing pregnancy. To explain this disparity between maternal Ob levels and placental transcript abundance they mentioned a variety of factors, such as the increasing mass of the syncytiotrophoblast, the stimulatory effect of estrogen on enhanced Ob production by maternal adipose tissue or the action of an Ob-binding protein. No Ob was found in the maternal circulation of pregnant *ob/ob* mice which were leptin-treated to be bred with wild-type males, and thus had placentas/fetal tissues with one intact Ob allele [168]. Kawai et al. attributed the source of hyperleptinemia in rat pregnancies rather to oversecretion by adipose tissue than to placental origin [170]. Zhao et al. published a comparative analysis of placental Ob production in mammals and wrote that the mechanisms leading to this increase may be species-specific with some species up-regulating adipose-derived Ob and some expressing Ob in the placenta [165]. Additionally, Gavrilova et al. [168] found in mice high levels of the soluble ObR-e which binds Ob with high affinity and prevents its renal clearance. Thus, the pregnancy-associated hyperleptinemia may be a compensatory mechanism to counteract leptin resistance, which might be caused by the inhibitory effect of leptin binding to ObR-e. In addition, the soluble ObR prevents passage of Ob through the blood-brain barrier [171] and therefore might be crucial in attenuating its satiety inducing effects in the hypothalamus during gestation, when energy demand is increased [172].

In conclusion, the physiological reasons for the pregnancy-related hyperleptinemia remain unclear. On the contrary, one would rather expect low maternal Ob levels with the effect of starvation, since the mother has to put great nutritional effort into the fetus and the preparation for lactation [159]. Hyperleptinemia as a compensatory mechanism to leptin resistance during pregnancy is one of many approaches discussed [159]. Another possibility is a more paracrine/autocrine action of Ob rather than a systemic one [159].

4.6.2.4.2. Leptin and the fetus

It has been hypothesized that Ob acts as *a fetal growth factor*. The fact that newborn *ob/ob* or *db/db* rodents are not distinguishable from wild-type littermates until 2 weeks after birth allows the conclusion that fetal Ob production is either not needed or is compensated by the maternal hormone [159]. Only 1.6-5% of Ob secreted *in vitro* by the term human placenta was estimated to reach the fetal circulation [173, 174]. Furthermore, no correlation was found between maternal Ob levels and fetal weight in humans by Schubring et al. [175], while umbilical cord blood Ob levels were positively associated with fetal birth weight and ponderal index by others [173, 176-179]. Nevertheless, it is difficult to distinguish whether these findings are the cause or the consequence of differences in fetal adipose mass [172]. Fetal fat tissue expresses Ob mRNA and protein but at lower levels than in adults [173]. Ob concentration is higher in umbilical veins compared to umbilical arteries, and it decreases rapidly during the neonatal period indicating that the human placenta may be one of the major sources of Ob in the fetal circulation [180]. Additionally, Ob was found to be produced also by fetal non-adipose tissues (lung, cartilage-bone, hair follicles, heart and liver) [69, 181, 182], where it may function in an autocrine/paracrine manner.

Indeed, Kirwin and her colleagues demonstrated *in vitro* and *in vivo* that Ob administration led to an increase in cellularity and percentage of type II alveolar cells expressing surfactant proteins in rat lung tissue [183]. These findings were further supported by experiments from another group indicating a direct role of Ob in enhancing surfactant production [182]. Thus Ob seems to play an essential role in regulating fetal lung maturation through pulmonary surfactant production by epithelial type II cells.

4.6.2.4.3. Uterine receptivity and implantation

Embryonic implantation is a crucial step in mammalian reproduction requiring perfect synchronization between embryo and maternal endometrium. There is ample evidence from studies performed in humans and rodents that Ob is involved in the embryo-maternal cross-talk and may have pivotal roles at the time of implantation.

To investigate Ob's role in the human implantation process, Kitawaki et al. [184] examined the expression of ObR and Ob in human endometrium. ObR mRNA and protein, but not Ob - neither mRNA nor protein - was present. Also, the changes in the amount of ObR mRNA were assessed in the course of the menstrual cycle: ObR mRNA expression was greatest in the early secretory phase, when the endometrium is preparing for acceptance of a fertilized egg.

Furthermore, Gonzalez and his colleagues [52] found the expression of Ob and ObR protein and mRNA in human endometrium by IHC and RT-PCR, respectively. The different potentials of arrested and competent blastocysts to secrete Ob during embryo-maternal crosstalk also implicate a role of Ob in this process.

Ob may promote implantation by acting on downstream molecules shown to be markers of endometrial receptivity. Endometrial expression of β3 integrin, an adhesion molecule as well as leukemia inhibitory factor, IL-1 and their receptors were higher in human endometrial epithelial cell cultures treated with Ob [185-187].

In a recent study by Dos Santos et al. [188], Ob expression by human endometria was compared between women suffering from recurrent implantation failure (IF) and fertile controls and was found to be significantly attenuated in the IF group. They suggest an involvement of Ob in this pathology and a possible new strategy for the treatment of IF in women. In the same study, ObR expression was found to be higher in endometria of women with IF than of fertile women.

Beside the work done in humans, Ob's role in the process of implantation was also examined in mice. Kawamura and co-workers suggest a paracrine/autocrine Ob signalling system in the uterus regulating the development of mouse pre-implantation embryos, as the addition of Ob to the embryo culture media significantly promoted pre-implantation embryonic development [189, 190]. Also, they detected Ob and ObR mRNA expression by means of nested PCR and RT-PCR, respectively in mouse blastocysts [190]. In the same study, Ob mRNA was identified in the mouse oviduct and uterus and Ob levels in the uterine fluid of pregnant mice were higher than those in non-pregnant mice [190]. It seems that Ob is required for normal implantation in mice. There was no pregnancy resulting, when exogenous Ob treatment of leptin deficient *ob/ob* mice was stopped 0.5 or 3.5 days post coitus, however, when Ob was ceased at later time points, it did not affect pregnancy [191]. On the other hand, in the study of Mounzih and co-workers withdrawal of exogenous Ob at any time points from Ob-deficient mothers resulted in normal term pregnancies and mice delivering normal-looking *ob/ob* offspring which died within one day after birth because of absence of lactation [192]. Ob and ObR expression was compared between implantation and inter-implantation sites in the mouse uterus on pregnancy day 4.5 and 5.5 (the time, when approaching and attachment of the embryo to the uterus take place) [193]. While Ob mRNA was not detected at all, ObR mRNA was expressed in the uterus and the blastocyst and the expression levels were lower in implantation compared to inter-implantation sites. In immunohistochemistry, both Ob and ObR were detected, but as Ob was not detected by RT-PCR, it might originate elsewhere [193]. The expression of ObR mRNA and protein was higher in the stroma than in the luminal epithelium (for RT-PCR, epithelial and stromal cells were separated by means of laser capture microdissection) [193]. Consistent with these findings, Alfer et al. did not find Ob mRNA, but detected the protein by IHC in human endometrial tissue [194]. In other works however, Ob mRNA was present in mouse as well as in human uterine tissue [2, 52, 189]. Yoon et al. [193] discuss that different PCR methods might be a reason for these contradictory results. The down-regulation of ObR expression at implantation sites is in agreement with a study by Reese et al., who identified molecular markers of uterine receptivity by global gene expression analysis and found ObR as one of the possible candidates [195]. The study by Yoon et al. [193] supports the suggestion of Dos

Santos et al. [188] that high ObR expression might be related to unsuccessful implantation. Yoon et al. propose embryonic estrogen as a direct regulator of endometrial ObR expression.

Although the involvement of Ob in the regulation of angiogenesis and expression of metalloproteinases is discussed elsewhere in this thesis, it is also important to mention here that both of these processes are essential for a successful implantation [5, 98, 191, 196].

4.6.2.4.4. Placental function

Castellucci and co-workers found Ob and ObR immunoreactivity in trophoblasts during all stages of human pregnancy [5]. Similar findings were obtained by Masuzaki [53] and Bodner [197]. Besides positive staining of trophoblasts, Castellucci et al. detected immunoreactivity also in endothelial cells of fetal vessels, amniotic and decidual cells [5]. The observed Ob and ObR in trophoblasts suggest a regulatory role of Ob and its receptor in placental physiology. Trophoblast cells from the embryonic trophectoderm invade the maternal endometrium in species where placentation is invasive like humans, rodents, and to some extent also in the dog. Trophoblast migration requires degradation of maternal extracellular matrix molecules by the activity of specific proteases. Metalloproteinases (MMPs) have been shown to be crucial factors for trophoblast migration and invasion [196]. *In vitro* Ob increased the secretion of MMP-2 and enhanced the activity of MMP-2 and MMP-9 (the gelatinase family of MMPs) from human cytotrophoblast cells [5, 198]. This indicates that Ob might be involved in the invasion of the trophoblasts by modulating the expression of MMPs [5]. Moreover, Gonzalez et al. found that Ob induces some alterations in the phenotype of human cytotrophoblasts increasing their invasive ability [198]. Furthermore, when introduced into choriocarcinoma cell cultures (a model for trophoblast development), Ob proved to be a trophic and mitogenic factor by means of inhibiting apoptosis and promoting proliferation in a dose- and time-dependent manner [3]. The potential involvement of Ob in trophoblast invasion is also suggested by the occurrence of abnormal Ob levels in preeclampsia, a pregnancy-complication associated with reduced trophoblast invasion [199].

Other functions of Ob in the placenta can be the regulation of placental and fetal growth, e.g. through the stimulation of placental angiogenesis [98] (which was described in more details earlier in this literature review) or through the regulation of nutrient transport. Leptin increased the activity of the system A amino acid transporter in human placental villous fragments in a concentration-dependent manner [200]. Furthermore, high-fat diet fed mice with increased circulating maternal leptin showed increased trans-placental transport of glucose and neutral amino acids associated with up-regulation of the protein expression of glucose and amino acid transporters in microvillous plasma membranes compared to a control group [201]. Surprisingly and on the contrary to their hypothesis, Farley et al. [202] found decreased activity of the system A amino acid transporter and decreased syncytiotrophoblast expression of ObR in cases of maternal obesity compared to a lean group. They suggest that the decreased placental amino acid transport encountered in the

setting of maternal obesity may be related to maternal hyperleptinemia and development of placental Ob resistance evidenced by ObR down-regulation. In the lean group however, placental amino acid uptake was significantly stimulated by Ob, similar to the findings of Jansson et al. [200].

In addition, placental Ob may also have a local immune modulatory role [203].

4.6.2.4.5. Myometrial function

From human medicine it is known that obese women with high leptin levels show an increased rate of post-dated pregnancies, a decrease in the rate of spontaneous preterm delivery [204], increased induction rates and increased caesarean section rates [205]. Taken together, these features suggest that an inhibitory role of Ob on uterine contractions may be possible. This hypothesis is supported by *in vitro* studies showing a cumulative inhibitory effect of Ob on both spontaneous and oxytocin-induced contractions on myometrial biopsies taken at the time of elective caesarean section [206, 207]. Because of this tocolytic effect, Ob may be considered in the future to be used pharmacologically in cases of threatened preterm labour [208]. Additionally, a recent study showed that the risk of atonic uterine haemorrhage increased rapidly with increasing body mass index (BMI) [209]. This can be attributed to the smooth muscle relaxing effect of Ob. Because of the high serum leptin levels in obese women, Wuntakal et al. recently proposed the early use of increasing concentrations of oxytocin in obese women during labour. This should help to overcome Ob's inhibitory actions in labour and thus achieve regular uterine contractions as well as reduce the risk of atonic postpartum haemorrhage [210]. Contradictory to these studies are the findings of Gavrilova: Ob deficient mice treated with leptin to establish pregnancy (treatment was discontinued after day 1 of gestation) showed no signs of labour [168].

4.6.3. Leptin in the dog

In the dog, the link between peripheral Ob levels and obesity/nutrition/energy metabolism has already been established [211]. However, Ob's role in canine reproduction is not yet clear and not widely studied.

Up to now, Ob and ObR in the dog have been detected in white adipose tissue [212, 213], in the ovary [6], mammary tissue [57], endometrial tissue [214], myocardium [215] and oral melanomas [216]. Iwase et al. showed the presence of canine Ob mRNA by Northern blot analysis in different adipose tissue depots, but could not find it in the heart, lung, liver, kidney, skeletal muscle, spleen, pancreas and adrenal gland of adult beagle dogs [213].

4.6.3.1. Non-reproductive processes

Obesity is a growing problem in the dog population with an estimated incidence of around 22-40% [217]. It may have detrimental effects on the health and longevity of the animals predisposing them to a wide range of obesity-related diseases including orthopaedic problems, diabetes mellitus, urinary disorders and cardiovascular diseases [217]. Therefore, Ob in dogs has mainly been investigated in energy metabolism-related aspects and not so much yet with regards to reproductive processes.

Iwase et al. developed the first method to quantify canine plasma Ob [213, 218]. They cloned Ob cDNA, produced canine recombinant leptin in Escherichia coli and used this as an antigen to immunize rabbits and obtain anti-canine Ob specific antibodies. These antibodies reacted to canine Ob much stronger than to Ob of mouse, rat or human origin. Subsequently, they developed an in-house sandwich ELISA and measured Ob plasma concentrations in a group of 13 healthy beagle dogs. The concentrations observed were in the range of 1.4-5.6 ng/ml and were thus similar to those reported in other species e.g. 1.5 ng/ml in lean mice [219], 7.5 +/- 9.3 ng/ml in normal weight humans [72], 3.47 +/- 0.5 ng/ml in mature mares [75].

While in humans and mice it was shown already in 1995 that plasma leptin concentration is positively correlated with body fat content [73], this correlation was demonstrated in dogs only in 2002 [220]. Ishioka and co-workers even denote leptin as a reliable marker of adiposity in dogs regardless of age, gender and breed [74]. However, as Ob plasma levels were increased after subcutaneous injection of dexamethasone (0.14 mg/kg), they state that Ob's diagnostic usefulness may be invalidated if an animal is under glucocorticoid treatment [221]. Similar results were obtained by Nishii et al., where i.v. administration of 0.1 mg/kg dexamethasone drastically increased Ob concentrations and enhanced the Ob peak in response to a meal [222]. Oral administration of prednisolone (1-2 mg/kg) however did not affect plasma Ob concentrations [222]. Yilmaz et al. describe a dose-dependent response of Ob levels to the i.m. administration of methylprednisolone with low doses (1 mg/kg) causing an increase and high doses (10 mg/kg) decreasing Ob concentrations [223]. Although the results of these studies are not consistent (which might be due to differences in glucocorticoid type, dosage, route of administration or individual variations), they all suggest that exo- or endogenous glucocorticoids may affect circulating Ob and should be considered when Ob levels in clinical patients are evaluated [211]. Beside glucocorticoids' influence on plasma Ob levels, also the administration of insulin or glucose seem to affect Ob levels, namely to increase them [224].

There was no difference in plasma Ob levels between obese and non-obese diabetic animals despite obvious differences in body condition score (BCS), and obese diabetic dogs also had significantly lower plasma concentrations than their non-diabetic obese counterparts [225]. According to Nishii et al., polyphagia observed in dogs with diabetes mellitus might be partly explained by decreased plasma Ob concentration [225].

Furthermore, Ishioka et al. found diurnal variations of Ob in regularly fed dogs associated with feeding-fasting cycles [224]. The postprandial peak occurred about 5-8 h and plasma Ob slowly decreased to basal levels 19-23 h after a meal. This meal-associated diurnal rhythm of plasma Ob is in accordance with findings in humans [226] and suggests that when judging serum Ob levels, the fed/fasted state has to be known [211].

Beagle dogs on a weight gain-inducing high-fat diet had increased Ob gene expression in visceral white adipose tissue and high plasma Ob concentrations [227]. Conversely, weight loss resulted in decreased plasma Ob levels [228]. Thus, monitoring Ob plasma concentrations could be a useful tool to ensure the efficacy of a restriction diet in a canine weight loss program [84]. However, when interpreting plasma concentrations, they point out the importance of gaining information about the moment of blood sample collection because of the possible influence of circadian rhythm, feeding state, exo- or endogenous corticosteroid, insulin, glucose administration, and thyroid gland activity [84].

The role of Ob has also been investigated in inflammatory/infectious conditions. Yilmaz et al. showed that Ob levels increased in response to intravenously administrated endotoxin in adult mongrel dogs [229]. They discuss in comparison with studies of other species that increased Ob levels may confer benefit in endotoxemia [230, 231], but may also be involved in associated clinical signs such as anorexia [89, 232] and fever [232].

In addition, serum Ob concentrations have shown to be increased in canine hypothyroidism compared to healthy control dogs [233], and this difference still remained after adjustment for BCS. Conversely, successfully treated hypothyroid dogs responded with a significant decrease of about 50% in their Ob levels, which was not observed in non-responders [234]. It is speculated that increased Ob is due, at least in part, to its reduced turnover and degradation, or to the stimulating role of Ob on the hypothalamic-pituitary-thyroid axis in response to low thyroid hormone concentrations, which is then amplified in hypothyroidism [233, 234].

Leptin resistance, characterized by a failure of elevated circulating Ob to counteract obesity, is a well-known feature in humans and rodents [114]. Mechanisms considered to be involved in this inappropriate response to high Ob levels are e.g. alterations in the transport of Ob across the blood-brain barrier or alterations in the ObR signalling cascade [235]. Although this condition has not been evaluated in dogs so far, it is speculated that leptin resistance may also be a potential cause of obesity in dogs [84].

Cytokines can either stimulate or inhibit tumour growth depending on their concentration and other environmental factors [216, 236]. Greene and colleagues investigated the expression of Ob in oral melanomas in dogs [216]. As in humans, Ob and ObR are expressed in melanoma cells and are thought of being a melanoma growth factor [237]. Ob was also found to be widely expressed in canine oral melanomas, but no statistically significant association was demonstrated between Ob staining percentage/intensity in immunohistochemistry and mitotic index [216]. Because of the small sample size in this

study, they could not determine whether Ob actually had an impact on tumour behaviour in oral melanomas or was simply an incidental finding [216]. Additionally, Ressel et al. [57] suggest Ob's involvement in the development of canine mammary tumours. Ob and ObR were localized in normal, hyperplastic and neoplastic mammary tissues, while their expression was negatively correlated with malignancy.

Furthermore, Ob's involvement in the development and progression of canine cardiac diseases and congestive heart failure (CHF) has been studied. Fonfara et al. [215] investigated Ob mRNA concentration in blood samples from dogs with CHF and Ob expression in myocardial samples of dogs with cardiac disease, and compared the findings to dogs without any cardiac abnormalities. Dogs with CHF had significantly higher Ob mRNA levels in the blood, while Ob expression in the myocardium varied with the type and severity of cardiac disease and with myocardial region. The results obtained indicate that Ob might play a role in canine cardiac diseases, but to assess its value as a prognostic marker, further investigations are needed.

As in humans [87] Ob has also been implicated in the immune response in dogs. In the study of Van de Velde et al. [238] the effect of long-term weight gain and stable obese body weight on immune function in beagle dogs was investigated. A transient impairment of T-lymphocyte function was observed in the end of a long-term weight gain period indicating that changes in energy balance during obesity development might alter immune function.

4.6.3.2. Reproduction

As already mentioned above, few studies have investigated the role of Ob in canine reproduction.

A sexual dimorphism in plasma Ob levels was observed also in dogs, however not consistently. In the study of Saleri et al. Ob concentrations were higher in females than in males (1.3-1.5 ng/ml in females vs. 0.5-0.6 ng/ml in males) [239], whereas Ishioka and co-workers did not find a gender-specific difference [74]. Saleri and her group [239] also investigated variations in Ob levels during the bitch's reproductive cycle and found a significant increase during estrus compared to pro- and diestrus. This is different from women, where peak Ob concentrations were found during the luteal phase coincident with maximal progesterone levels [162]. Another interesting finding showing differences between women and dogs is the change in leptin levels triggered by reproductive senescence and gonadectomy, respectively. While leptin concentrations in bitches and spayed dogs did not differ, premenopausal women had higher leptin levels compared to postmenopausal individuals [126, 240].

Positive signals for Ob and ObR were detected by immunohistochemistry in prepubertal and adult non-pregnant bitches in various cells of the ovary during different stages of the cycle,

suggesting a regulatory role for Ob in canine ovarian function. However, the phase of the CL was not specified and ObR was not found in the CL [6].

Another study conducted by our group [7] examined the temporal pattern of Ob and ObR expression in the canine CL in diestrus and during pregnancy as well as after aglépristone induced luteolysis. Both Ob and ObR mRNA were present at all stages investigated with a significant increase in Ob in the mid-luteal phase of non-pregnant bitches. As this up-regulation coincides with generally high serum progesterone levels, this finding may indicate a possible role for Ob in CL formation and steroid production. These findings are in accordance with Kumar et al. [106], who obtained similar results with Ob and ObR mRNA levels in bovine CLs being highest in mid and late luteal stages in non-pregnant buffalo cows [106].

Furthermore, a recent study by Bartel et al. [214] investigated Ob and ObR expression by IHC in endometrial tissue of non-pregnant dogs with particular attention to the so-called foamy endometrial epithelial cells (fat-accumulating cells which occur physiologically during late metestrus of the canine reproductive cycle [29, 37, 241, 242]). Signals for both proteins were found in the foamy endometrial epithelial cells as well as in those without lipid-accumulation. The authors hypothesized that lipid-accumulation within these endometrial cells is not a sign of degeneration, but rather has a potential function during canine pregnancy in preparing the uterine environment for placentation.

5. Objectives

The aim of this study was to investigate whether Ob might be involved in the establishment and maintenance of pregnancy in the dog. Therefore, we determined the localization of Ob and ObR at the mRNA and protein level by ISH and IHC, respectively, in the CL, uterus and placenta of pregnant bitches as a preliminary step for Ob's and its cognate receptor's functional characterization. Furthermore, semi-quantitative RT-PCR was performed to investigate changes in gene expression levels of Ob and ObR over the course of pregnancy. In addition, uterine Ob and ObR mRNA concentration was compared between non-pregnant dogs in early diestrus (E-) and dogs in the pre-implantation period (E+).

6. Materials and methods

6.1. Animals and collection of tissue

All animal experiments were carried out in accordance with animal welfare legislation (permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c20/15c GI 18, 14 (Giessen) and permit no. Ankara 2006/06 (Faculty of Veterinary Medicine, University of Ankara)).

Experiment 1: 18 healthy bitches (2-8 years, different breeds) were mated 2 days after ovulation (P4 ≥ 5 ng/ml) by a fertile male and ovariohysterectomized on the following days of pregnancy:

Group 1: days 8-12, pre-implantation; n=5

Group 2: days 18-25, post-implantation; n=5

Group 3: days 35-40, mid-gestation; n=5

Group 4: during prepartum luteolysis; n=3

The day of mating was recorded as day 0. In the pre-implantation group, pregnancies were confirmed by detecting embryos in uterine flushes. To identify the time of prepartum luteolysis, P4 levels were monitored at 6 h intervals from day 58 of pregnancy onwards. When P4 continued to decline below 3 ng/ml in two consecutive measurements, the bitches were ovariohysterectomized.

Experiment 2: To evaluate whether leptin signalling is involved in embryo-maternal communication in the uterus during early pregnancy, Ob and ObR mRNA expressions were compared between non-pregnant dogs in early diestrus and pregnant dogs in the pre-implantation period. Timing of ovulation and matings were as in Experiment 1. All dogs were mated and were ovariohysterectomized 8-12 days after mating. Bitches with embryos identified in uterine flushes were grouped as E+ (n=8), while bitches with no detectable embryos served as negative controls (E-; n=6).

The tissues obtained were CL, full-thickness utero-placental sections from placental sites and inter-placental uterine sections from free polar zones. They were treated as follows:

For immunohistochemistry (IHC) and in situ hybridization (ISH), tissue samples i.e. CL, pre-implantation uteri, inter-placental uterine sections and utero-placental units of experiment 1, were trimmed off surrounding connective tissue and fixed for 24 h at 4°C in 10% neutral phosphate-buffered formalin immediately after their removal. After that, they were washed in frequently changed phosphate buffered saline (PBS) during one week, dehydrated in a graded ethanol series and embedded in paraffin-equivalent Histo-Comp (Vogel, Giessen, Germany).

For RNA preservation, tissue samples i.e. pre-implantation uteri, placental uterine sections and utero-placental units of experiment 1, and pre-implantation uteri as well as matched early diestrus uteri of experiment 2 were placed in RNAlater (Ambion Biotechnologie GmbH, Wiesbaden, Germany) immediately after surgery and incubated for 24 h at 4°C. Long-term storage was at -80°C until analysis.

In the prepartum luteolysis group no inter-placental uterine sections were available. Luteal Ob and ObR mRNA expression was previously described by our group [7].

6.2. Immunohistochemistry (IHC)

First, all tissue samples were cut (2-3 µm thick) with a microtome and sections were mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Afterwards, slides were deparaffinized in xylol, rehydrated in a graded ethanol series and washed under running tap water (5 min). Antigen retrieval was achieved by heat induction incubating slides in 10 mM citrate buffer (pH 6.0) at room temperature for 5 min followed by 15 min at 100°C in a microwave oven. Then slides were cooled at room temperature for 20 min and washed under running tap water for 5 min. Endogenous peroxidase activity was quenched by incubating slides in 0.3% hydrogen peroxide in methanol for 30 min on a shaker. Subsequently, they were rinsed in IHC buffer/0.3% Triton X (pH 7.2-7.4; composition see in appendix) for 5 min and then placed in coverplates. Non-specific binding sites were blocked by incubating sections with 10% goat serum (for Ob; KPL, Gaithersburg, USA) or 10% horse serum (for ObR; Vector Laboratories, Burlingame, USA) for 20 min at room temperature. Slides were incubated overnight at 4°C with one of the following primary antibodies.

- anti-Ob antibody: rabbit polyclonal affinity purified IgG directed towards the N-terminal region of the human Ob (ARP41697_P050, Aviva Systems Biology, San Diego, USA) diluted 1:200 in IHC buffer/0.3% Triton X. This antibody has not been tested in canine tissues before and was chosen based on a predicted 85% homology of the immunogen sequence with the dog.
- anti-ObR antibody: goat polyclonal affinity purified IgG raised against a peptide mapping at the C-terminus of the short form of ObR of mouse origin (Ob-R (M-18): sc-1834, Santa Cruz Biotechnology Inc., CA, USA) diluted 1:50 in IHC buffer/0.3% Triton X. This antibody is recommended for the detection of both short and long ObR isoforms in several species including the dog.

Negative controls were either incubated with IHC buffer/0.3% Triton X or pre-immune rabbit (for Ob) and goat (for ObR) IgG (Vector Laboratories, Burlingame, USA) instead of the primary antibody. The following positive controls were used:

- Ob: mouse ovary and pituitary
- ObR: canine pituitary and subcutaneous adipose tissue

The next day, after washing 2x 5 min with IHC buffer/0.3% Triton X, tissue sections were incubated for 30 min at room temperature with 100 µl of the 1:100 diluted secondary biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, USA) and horse anti-goat antibody (Vector Laboratories, Burlingame, USA) for Ob and ObR detection, respectively. After washing again for 5 min with IHC buffer/0.3% Triton X, slides were incubated for 30 min with avidin/biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, USA) followed by washing for 5 min in IHC buffer/0.3% Triton X. Positive signals

were detected using the Liquid DAB+ substrate Kit (Dako Schweiz AG, Baar, CH). The enzyme reaction was stopped after 5, 10 or 20 min by dipping the slides into tap water. Lastly, sections were slightly counterstained with haematoxylin, washed under running tap water, dehydrated in a graded ethanol series and covered with coverslides.

6.3. In situ hybridization (ISH)

A non-radioactive method was used for the tissue localization of Ob and ObR mRNA according to the previously described protocol [17]. The following canine-specific primers were used in qualitative PCR to create templates for subsequent cRNA probe synthesis:

- Ob forward: ATG CGT TGT GGA CCT CTG TG
- Ob reverse: GGT TGG AGC CCA GGA ATG AA
- ObR forward: CAT GGT GGG TGA CCG TGT TA
- ObR reverse: TCC CTC GAG TGA TTG GAT TGC

The length of the amplicon was 203bp and 232bp for Ob and ObR, respectively. PCR products were separated on a 2% ethidium bromide-stained agarose gel, purified by means of the Qiaex II gel extraction system (Qiagen GmbH, Hilden, Germany) and cloned into the pGEM-T plasmid (Promega). Digestion of the pGEM-T plasmid clones containing the respective inserts was performed with the restriction enzymes *Nco*I (antisense cRNA) and *Not*I (sense cRNA) (New England Biolabs, Frankfurt, Germany). Synthesis of digoxenin (DIG)-labelled cRNA probes was accomplished using the DIG-RNA labelling kit (Roche Diagnostics AG, Rotkreuz, CH). Semi-quantitation of the labelled probes was achieved by dot blot analysis of serial dilutions of DIG-labelled cRNA on a positively charged nylon membrane (Roche Diagnostics AG, Rotkreuz, CH).

Paraffin-embedded cross sections of CL, uterus at the inter-placental free polar zone and utero-placental unit were cut with a microtome (2-3 µm thick), mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), dewaxed, rehydrated, digested with 70 µg/ml proteinase K (Sigma-Aldrich Chemie GmbH) for permeabilization, post-fixed with 4% paraformaldehyde and then processed according to the procedure by Lewis and Wells [243] and Klonisch et al. [244]. The hybridization was performed overnight at 37°C. The DIG-labelled cRNA probes were detected by means of alkaline phosphate conjugated sheep anti-DIG Fab fragments (Roche Diagnostics AG, Rotkreuz, CH) diluted 1:5000 in 1% ovine serum as recommended by the supplier. Signals were detected by incubating the sections with the substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitroblue tetrazolium (NBT/BCIP; Roche Diagnostics AG, Rotkreuz, CH). As negative controls, sense probes were used.

Representative samples for Ob detection in the CL and inter-placental uterine section at post-implantation, and in the utero-placental unit at prepartum luteolysis were selected. Signals for ObR mRNA in the inter-placental uterine section and utero-placental unit were

evaluated from representative samples of the post-implantation and prepartum luteolysis groups, respectively.

6.4. Semi-quantitative Real Time (TaqMan) PCR

Total RNA was isolated from tissues (pre-implantation uterus and inter-placental uterine sections as well as utero-placental units) using the Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). Samples were DNase-treated (RQ1 RNase-free DNase; Promega, Dübendorf, CH) in order to remove genomic DNA contamination. To obtain cDNA, reverse transcription was carried out in an Eppendorf Mastercycler® (Vaudaux-Eppendorf AG, Basel, CH) using 100-200 ng RNA from each tissue sample for Ob and ObR. The following primers and probes that were previously designed based on known canine leptin (GenBank accession number NM_001003070) and leptin receptor (long form, GenBank accession number NM_001024634) sequences and already used in another study [7] were also applied here: Ob (forward): 5'-GGG TCG CTG GTC TGG ACT T-3', Ob (reverse): 5'-CTG TTG GTA GAT GGC CAA CGT-3', Ob TaqMan Probe: 5'-TCC TGG GCT CCA ACC AGT CCT GAG T-3', amplicon length 86 bp; ObR (forward): 5'-CAT TTG CGG AGG GAT GGT T-3', ObR (reverse): 5'-AGC GGT TTC ACC ACG GAA T-3', ObR TaqMan Probe: 5'-TTG ACT CTT CAC CAA CGT GTG TGG TTC C-3', amplicon length 149 bp. TaqMan probes were labelled at the 5'-end with the reporter dye 6-carboxyfluorescein (FAM), and at the 3'-end with the quencher 6-carboxytetramethylrhodamine (TAMRA). Semi-quantification of Ob and ObR mRNA expression in the uteri and utero-placental sections was performed by Real Time (TaqMan) PCR analysis according to our protocols [245] in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using 96-well optical plates (Applied Biosystems, Foster City, CA, USA). Samples were run in duplicates and autoclaved water instead of cDNA served as negative control. As reference genes, canine GAPDH (GenBank: AB028142; GAPDH (forward): 5'-GCT GCC AAA TAT GAC GAC ATC A-3', GAPDH (reverse): 5'-GTA GCC CAG GAT GCC TTT GAG-3', GAPDH TaqMan Probe: 5'-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3'; [246]) and canine cyclophyllin A (Prod. No. Cf03986523-gH, Applied Biosystems, Foster City, CA, USA) were used.

6.4.1. Statistical analysis

Data from the semi-quantitative Real Time (TaqMan) PCR was considered valid when the relative amounts of reference genes for a sample were constant (i.e. similar in the duplicate samples), and their average amount was used for the calculation of relative gene expression (RGE) based on the comparative ΔΔCt method according to the previously described protocol [246]. Logarithmic transformation was performed when data was not normally distributed (Kolmogorov–Smirnov test, $P > 0.05$). A parametric one-way ANOVA followed by Tukey HSD was used to compare gene expression levels between pregnancy stages in experiment 1. In experiment 2, a two-sided t-test for independent samples was performed.

(As the expression level of Ob was below the detection limit in the E- group, no statistical analysis was performed for Ob.) Results are presented as mean ± SE of observed or logarithmically transformed data. Level of significance was set at $P < 0.05$. All statistical tests were carried out with IBM® SPSS® Statistics for Windows, Version 19.0 (Armonk, NY, USA).

7. Results

7.1. Immunohistochemistry

7.1.1. Corpus luteum

7.1.1.1. Leptin

Immunostaining was detected in the cytoplasm of luteal cells during all stages of pregnancy with subjectively strongest signals in the post-implantation group. In contrast to ObR, the cytoplasmic staining of luteal cells was evenly distributed and homogenous throughout the whole cell. Additionally, in the mid-gestation and prepartum luteolysis groups, endothelial cells stained positive within the CL. Fibroblasts within the CL showed no immunoreaction for Ob (Figures 5-8).

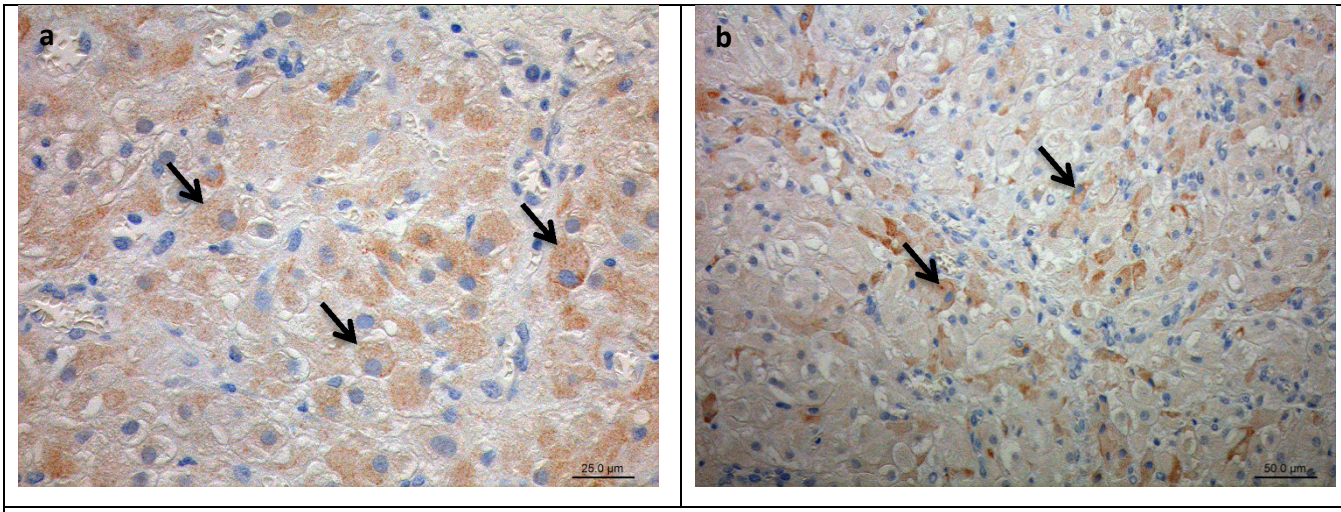


Figure 5: CL of the pre-implantation group with positive staining of the luteal cells (black arrows) (a&b)
(a: scale bar = 25 µm; b: scale bar = 50 µm)

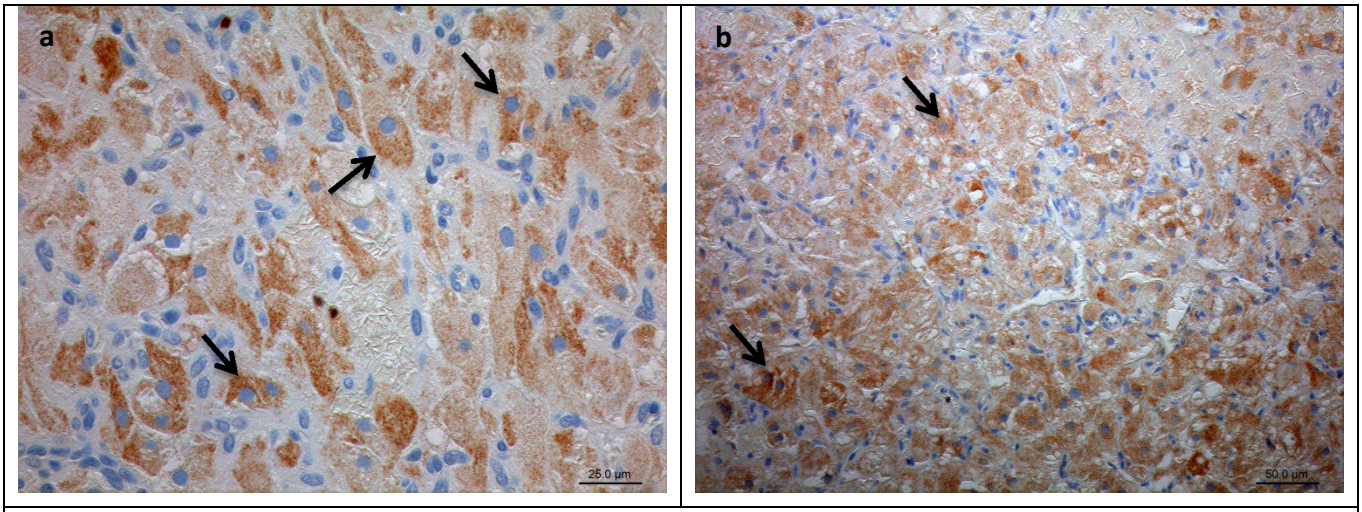


Figure 6: CL of the post-implantation group with strong positive staining of the luteal cells (black arrows) (a&b) (a: scale bar = 25 µm; b: scale bar = 50 µm)

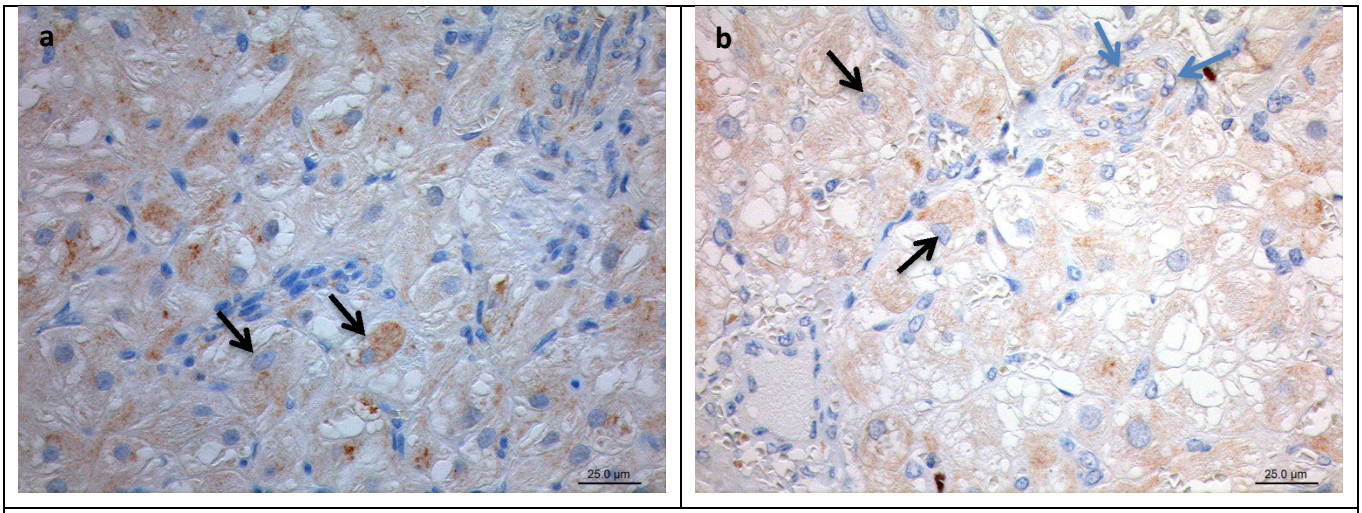


Figure 7: CL of the mid-gestation group with positive staining of the luteal (black arrows) and endothelial (blue arrows) cells (a&b) (a&b: scale bar = 25 µm)

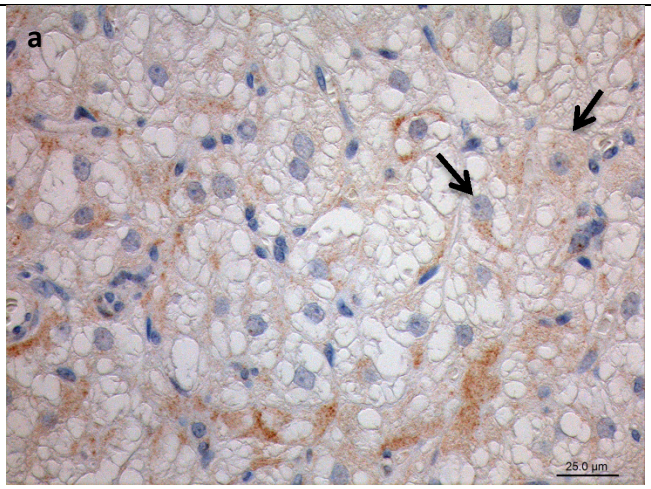


Figure 8: CL of the prepartum luteolysis group with positive staining of the luteal cells (black arrows) (a: scale bar = 25 µm)

7.1.1.2. Leptin receptor

ObR was detected in the luteal and endothelial cells of the CL throughout the whole pregnancy. In contrast to Ob, the cytoplasm of luteal cells was rather heterogeneously stained with a weaker diffuse and a stronger granular staining pattern. Fibroblasts within the CL did not show immunoreactivity (Figure 9).

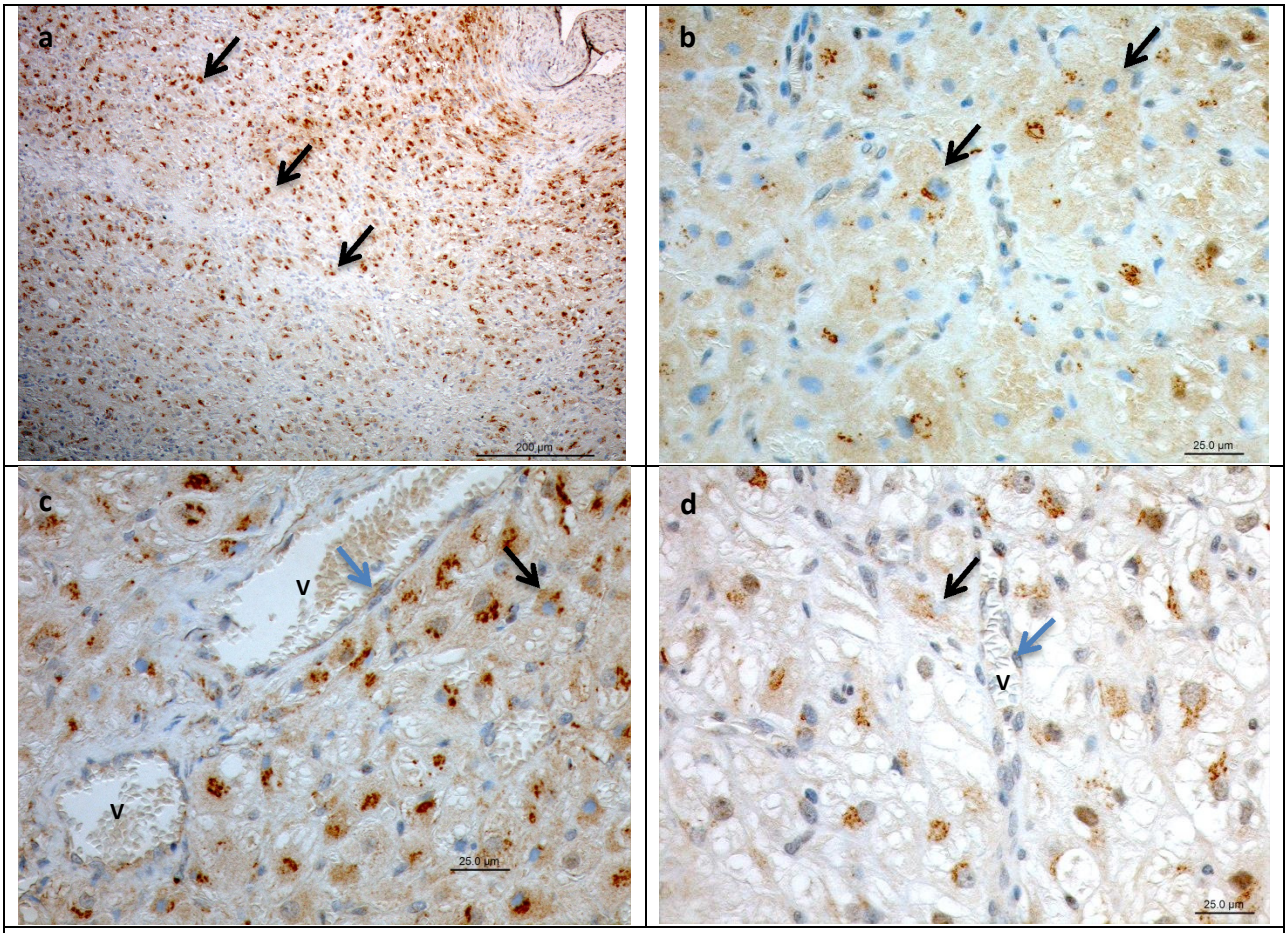


Figure 9:

a) CL of the pre-implantation group: black arrows indicate luteal cells (scale bar = 200 µm)
b) CL of the post-implantation group: black arrows indicate luteal cells (scale bar = 25 µm)
c) CL of the mid-gestation group: black arrow indicates luteal cell and blue arrow indicates endothelial cell (V=vessel) (scale bar = 25 µm)
d) CL of the prepartum luteolysis group: black arrow indicates luteal cell and blue arrow indicates endothelial cell (V=vessel) (scale bar = 25 µm)

7.1.2. Pre-implantation uterus and inter-placental uterine sections

7.1.2.1. Leptin

In all stages examined (pre-implantation, post-implantation and mid-gestation), positive signals for Ob were detected in the epithelial cells of the luminal lamina epithelialis, the superficial and deep glands. Immunostaining was also present in the myometrial muscle cells and in smooth muscle cells of vessels. Endothelial cells and fibroblasts showed sporadic positive reaction (Figure 10).

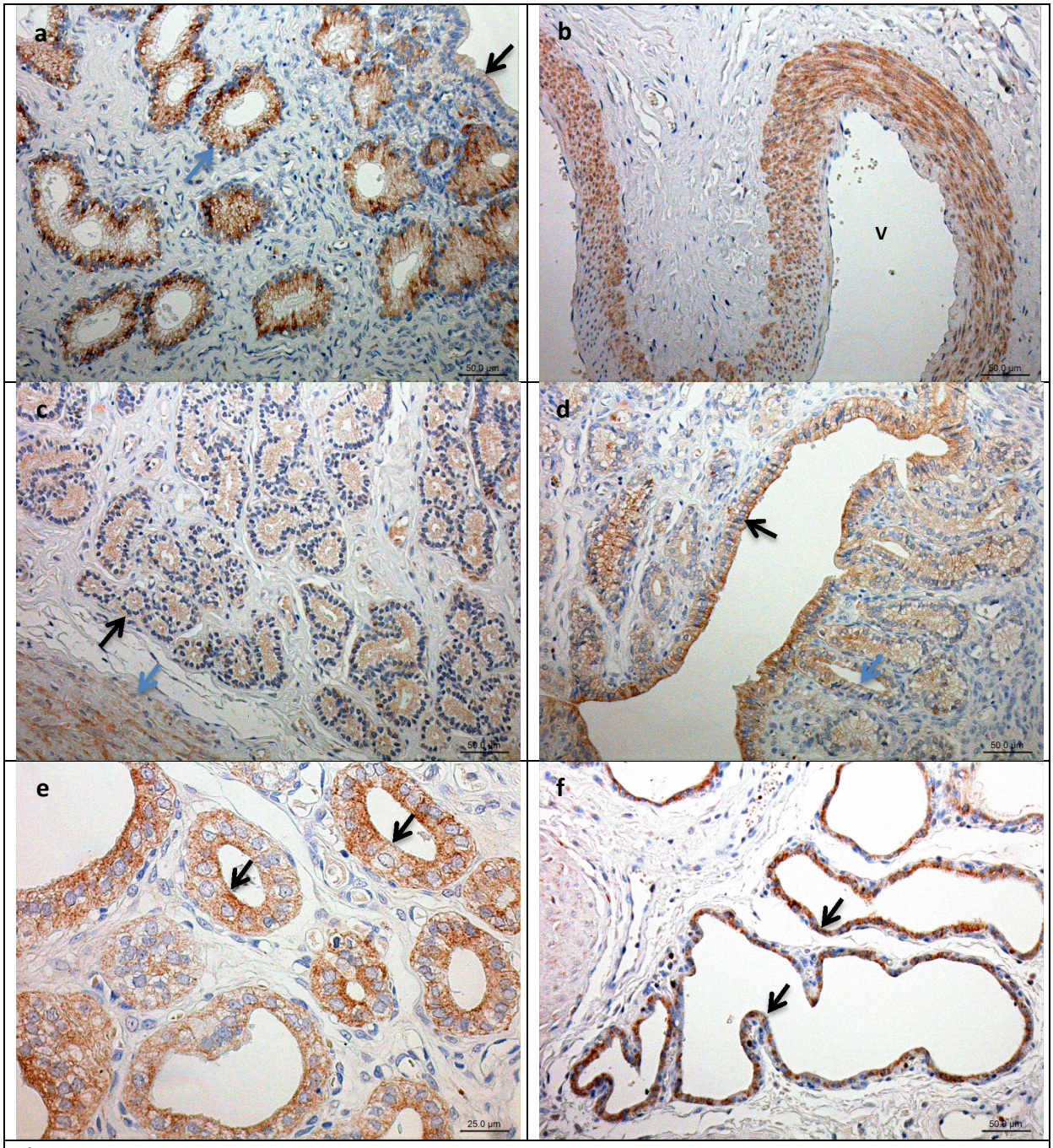


Figure 10:

a) Epithelial cells of lamina epithelialis (black arrow) and superficial glands (blue arrow) staining positive for Ob (pre-implantation group) (scale bar = 50 µm)

b) Vessel (V) within the stratum vasculare of the myometrium showing positive staining for Ob within the smooth muscle cells of blood vessel wall (pre-implantation group) (scale bar = 50 µm)

c) Epithelial cells of deep uterine glands (black arrow) and myometrial muscle cells (blue arrow) staining positive for Ob (post-implantation group) (scale bar = 50 µm)

d) Luminal (black arrow) and glandular (blue arrow) epithelial cells with positive staining for Ob (post-implantation group) (scale bar = 50 µm)

e) Epithelial cells of superficial uterine glands (black arrows) staining positive for Ob (mid-gestation group) (scale bar = 25 µm)

f) Epithelial cells of dilated deep uterine glands (black arrows) staining positive for Ob (mid-gestation group) (scale bar = 50 µm)

7.1.2.2. Leptin receptor

ObR was also detected throughout all stages examined (pre-implantation, post-implantation and mid-gestation) in the epithelial cells of the endometrial lamina epithelialis (especially in the apical cytoplasmic region) and the superficial and deep glands. There were also prominent stromal signals coming from the endometrium. Myometrial muscle cells, smooth muscle cells of blood vessel media, myometrial fibroblasts and sporadically endothelium also stained positive (Figure 11).

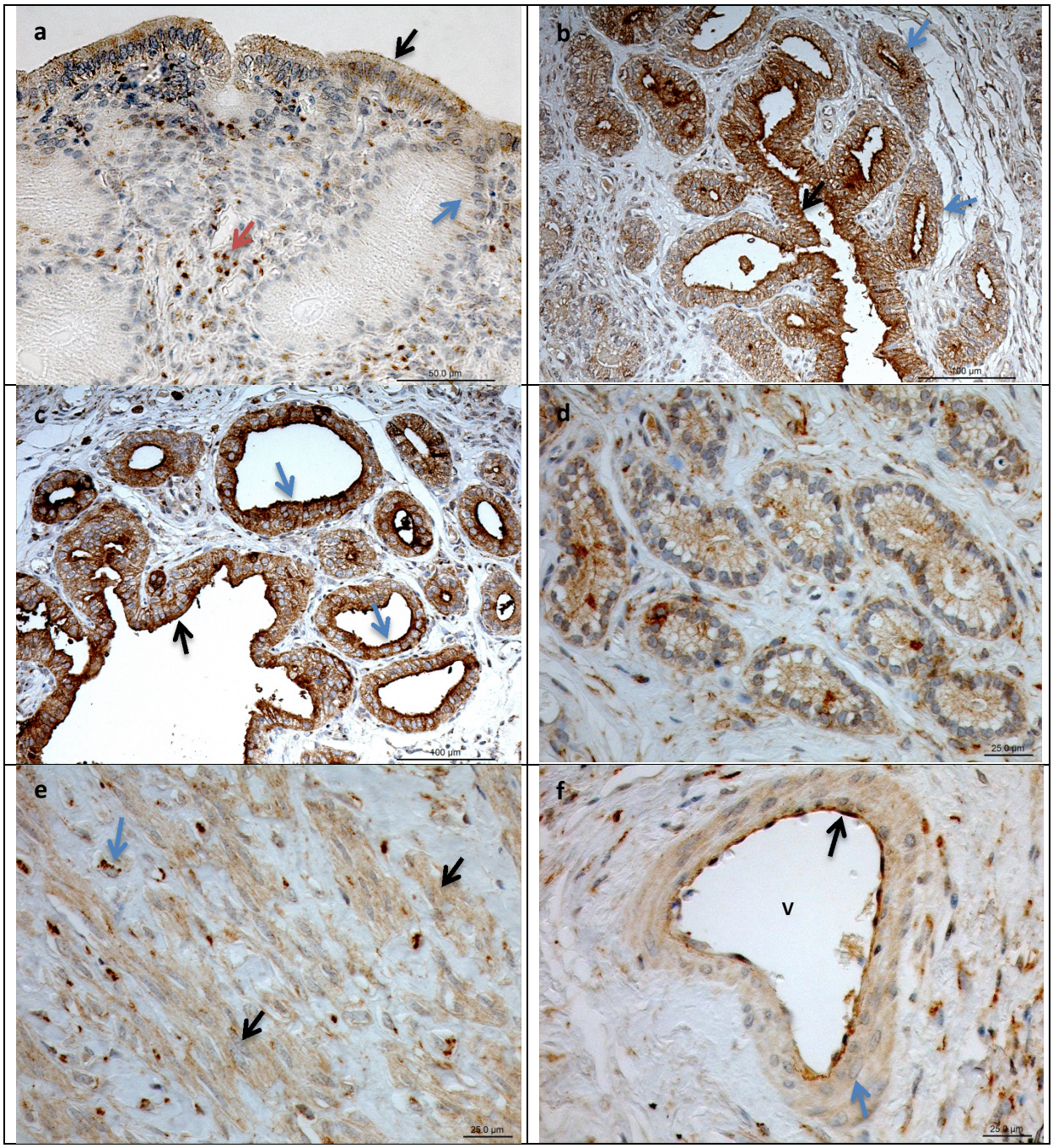


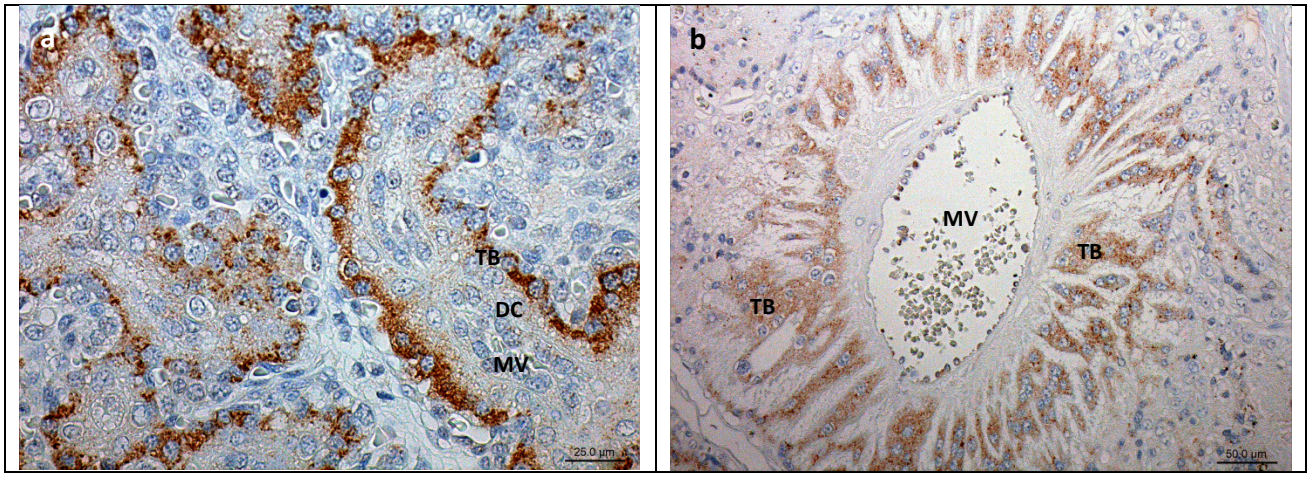
Figure 11:

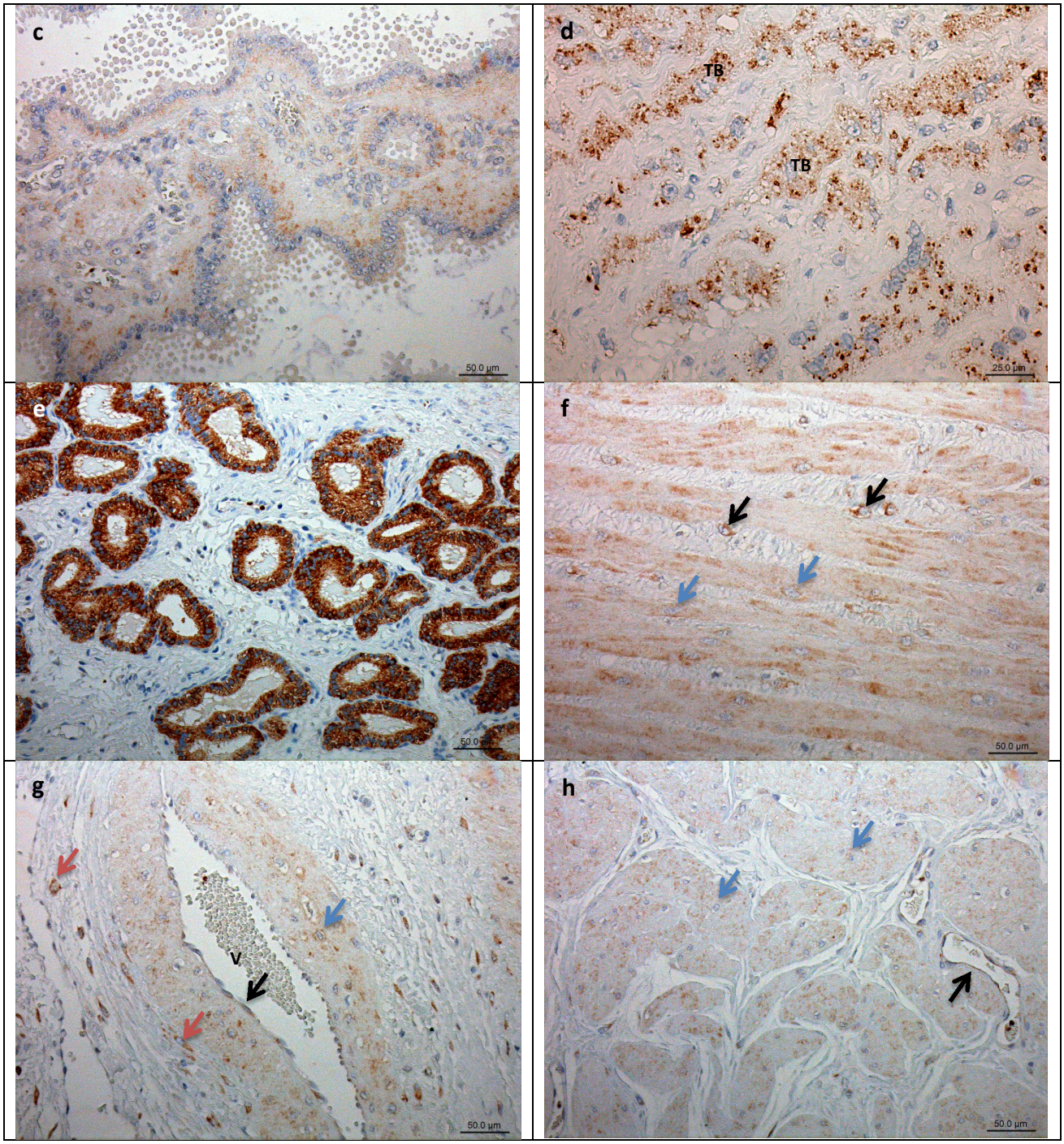
a) Immunostaining present in epithelial cells of the lamina epithelialis (black arrow) and superficial glands (blue arrow) and endometrial stroma (red arrows) (pre-implantation group) (scale bar = 50 µm)
b) Positive signals in the epithelial cells of the endometrial lamina epithelialis (black arrow) and the superficial glands (blue arrows) (especially in the apical cytoplasmic region) (post-implantation group) (scale bar = 100 µm)
c) Positive staining for ObR in the luminal (black arrow) and glandular (blue arrows) epithelial cells with more intense staining in the apical region of the cells (mid-gestation group) (scale bar = 100 µm)
d) Positive staining for ObR in the epithelial cells of the deep uterine glands (mid-gestation group) (scale bar = 25 µm)
e) Weak staining of the muscle cells (black arrows) and strong punctual staining of the fibroblasts (blue arrow) within the stratum circulare of the myometrium (mid-gestation group) (scale bar = 25 µm)
f) Vessel (V) within the stratum vasculare of the myometrium showing positive signal for ObR in the endothelial (black arrow) and smooth muscle cells (blue arrow) as well as in the surrounding fibroblasts (mid-gestation group) (scale bar = 25 µm)

7.1.3. Utero-placental unit

7.1.3.1. Leptin

In the placental labyrinth fetal trophoblasts showed strong immunoreactivity, while staining in the decidual cells was weak. Invading trophoblasts surrounding large maternal vessels at the base of the labyrinth had prominent positive signals. In the glandular chambers epithelial as well as some stromal cells showed immunostaining. Furthermore, positive signals were detected in the cover layer of endometrial connective tissue, in the epithelial cells of deep uterine glands, myometrial muscle cells and fibroblasts and in blood vessel smooth muscle layer and some endothelial cells (Figure 12).




c
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TB
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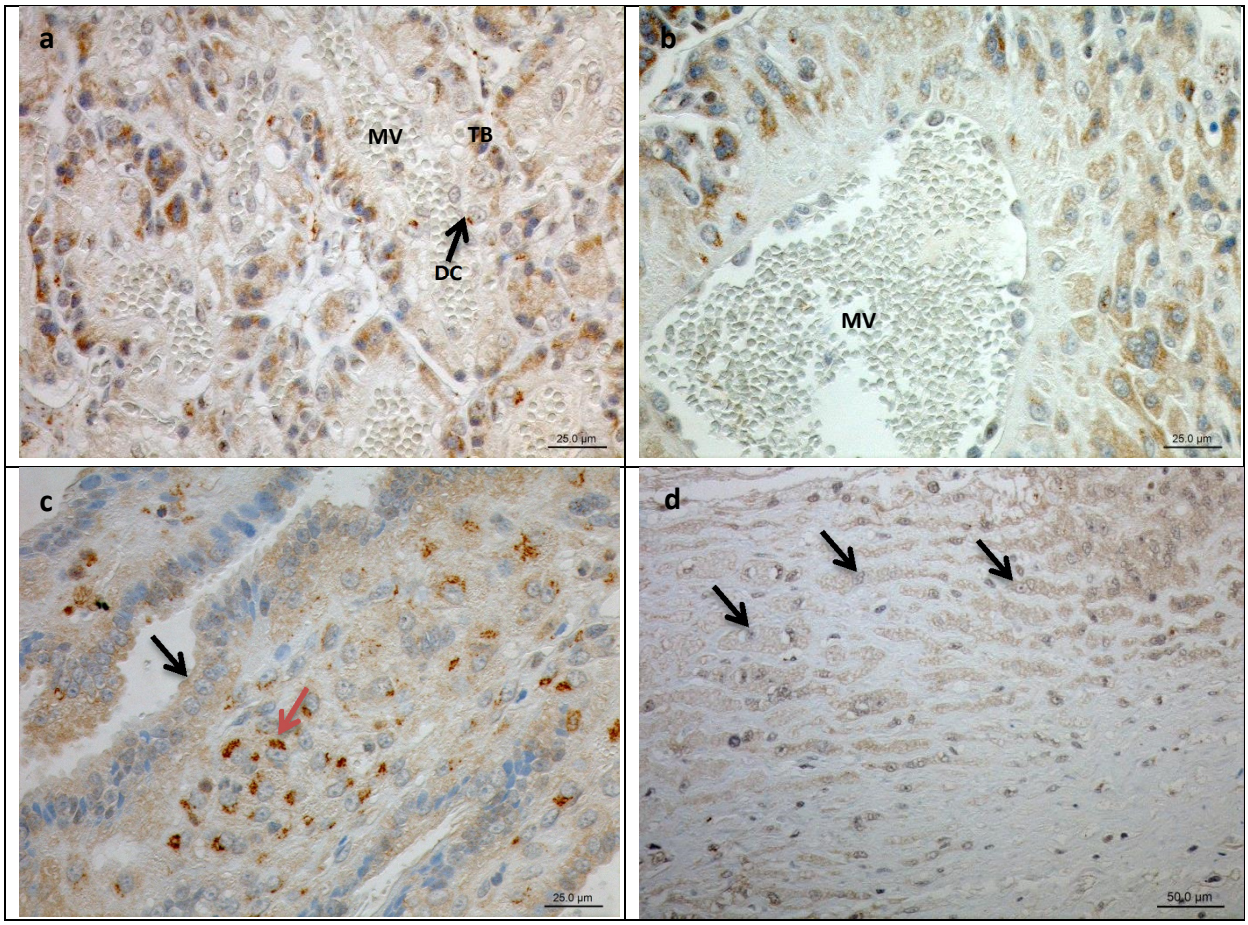
Figure 12:

a) Placental labyrinth: maternal vessels (MV) surrounded by weakly stained decidual cells (DC) and fetal trophoblasts (TB) with intense positive staining (mid-gestation group) (scale bar = 25 µm)
b) Large maternal vessel (MV) at the base of the placental labyrinth surrounded by positively stained invading trophoblasts (TB) (prepartum luteolysis group) (scale bar = 50 µm)
c) Glandular chambers with weak positive staining in the epithelial cells (mid-gestation group) (scale bar = 50 µm)
d) Cover layer of endometrial connective tissue invaded by positively stained trophoblasts (TB) (prepartum luteolysis group) (scale bar = 25 µm)
e) Deep uterine glands with strong positive staining in the epithelial cells (post-implantation group) (scale bar = 50 µm)
f) Stratum circulare of the myometrium with positively stained muscle cells (blue arrows) and interjacent fibroblasts (black arrows) (prepartum luteolysis group) (scale bar = 50 µm)
g) Vessel (V) within the myometrial stratum vasculare showing positive signals in the endothelial (black arrow) and smooth muscle cells (blue arrow) as well as in the surrounding fibroblasts (red arrows) (prepartum luteolysis group) (scale bar = 50 µm)
h) Stratum longitudinale of the myometrium with positively stained muscle (blue arrows) and endothelial cells (black arrow) (prepartum luteolysis group) (scale bar = 50 µm)

7.1.3.2. Leptin receptor

The results of the IHC in the utero-placental unit for ObR protein detection show a similar distribution pattern to Ob protein (see above). In general, endometrial and myometrial stromal signals were more prominent for ObR compared to Ob (Figure 13).



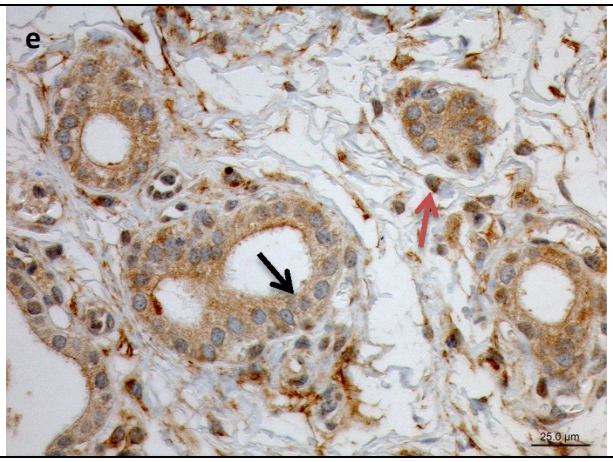
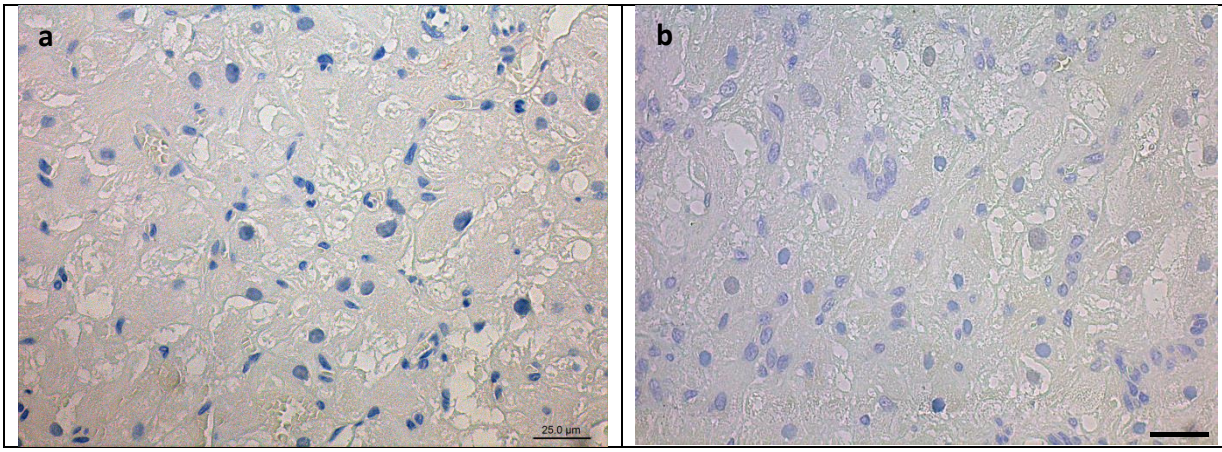


Figure 13:

a) Placental labyrinth: maternal vessels (MV) surrounded by weakly stained decidual cells (DC) and fetal trophoblasts (TB) with intense positive reaction (prepartum luteolysis group) (scale bar = 25 µm)
b) Large maternal vessel (MV) at the base of the placental labyrinth surrounded by positively stained invading trophoblasts (prepartum luteolysis group) (scale bar = 25 µm)
c) Glandular chambers with positivity for ObR in the epithelial cells (black arrow) and strong stromal signals (red arrow) (post-implantation group) (scale bar = 25 µm)
d) Cover layer of endometrial connective tissue invaded by positively stained trophoblasts (black arrows) (prepartum luteolysis group) (scale bar = 50 µm)
e) Deep uterine glands with immunostaining for ObR in the epithelial cells (black arrow) and the surrounding endometrial fibroblasts (red arrow) (mid-gestation group) (scale bar = 25 µm)

7.1.4. Isotype controls

There was no immunostaining in any of the isotype controls (Figure 14).



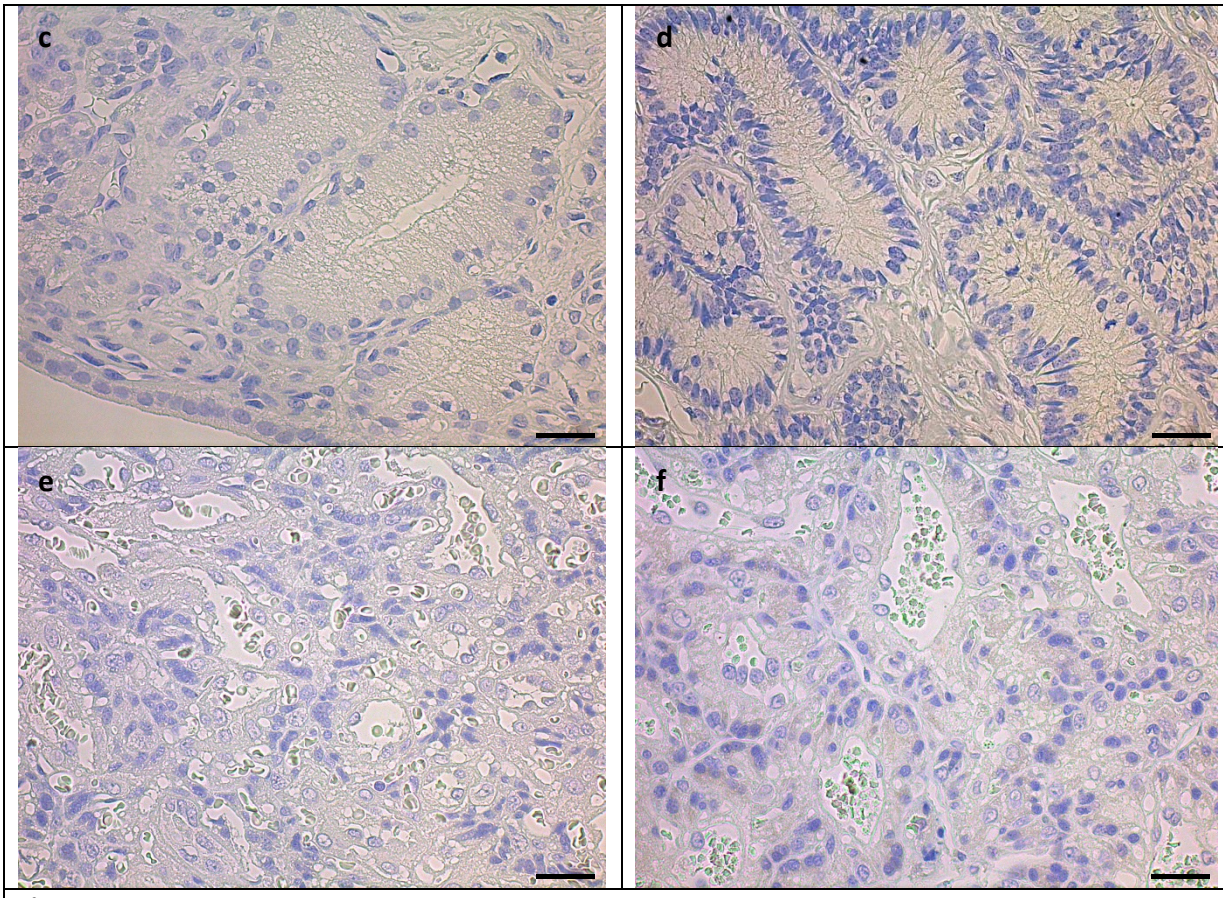


Figure 14:

a) CL at post-implantation, Ob (scale bar = 25 µm)
b) CL at post-implantation, ObR (scale bar = 25 µm)
c) Uterus at pre-implantation, Ob (scale bar = 25 µm)
d) Uterus at pre-implantation, ObR (scale bar = 25 µm)
e) Utero-placental section at prepartum luteolysis, Ob (scale bar = 25 µm)
f) Utero-placental section at prepartum luteolysis, ObR (scale bar = 25 µm)

7.1.5. Positive controls

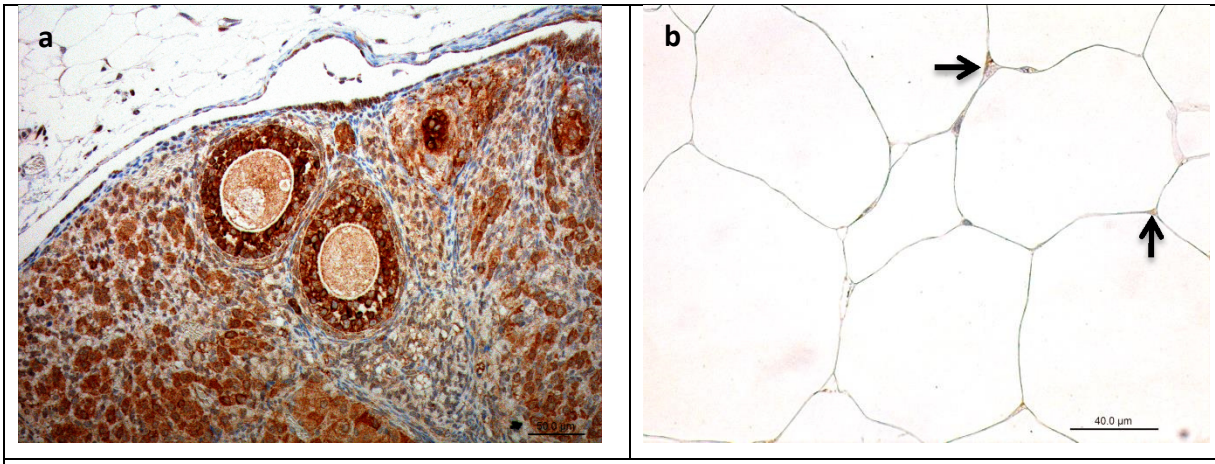


Figure 15:

a) Mouse ovary, Ob: luteal cells, granulosa cells and oocytes stained positive (scale bar = 50 µm)
b) Dog subcutaneous fat, ObR: positive signals are indicated with arrows (scale bar = 40 µm)

7.2. In situ hybridization

A similar localization pattern was observed for Ob and ObR at the mRNA level by ISH in all tissues examined like with IHC at the protein level.

7.2.1. Corpus luteum

7.2.1.1. Leptin

In the CL, Ob mRNA was detected in the cytoplasm of luteal and endothelial cells in the post-implantation group (Figure 16).

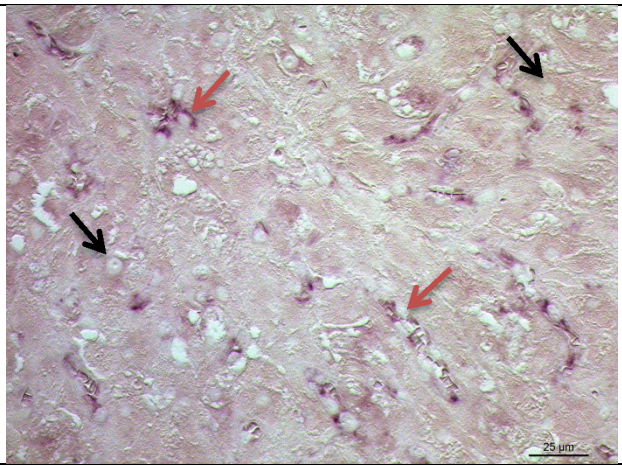


Figure 16: Positive signals for Ob mRNA in the cytoplasm of luteal (black arrows) and endothelial cells (red arrows) in the post-implantation group (scale bar = 25 µm)

7.2.2. Inter-placental uterine sections

7.2.2.1. Leptin

In the inter-placental uterine sections of the post-implantation group, Ob mRNA was found in the epithelial cells of the lamina epithelialis, the superficial and deep uterine glands. Furthermore, signals were detected in myometrial smooth muscle cells, in myometrial fibroblasts as well as vessel endothelium and smooth muscle layer. In the fibroblasts and myometrial smooth muscle cells, staining was more pronounced in the perinuclear region (Figure 17).

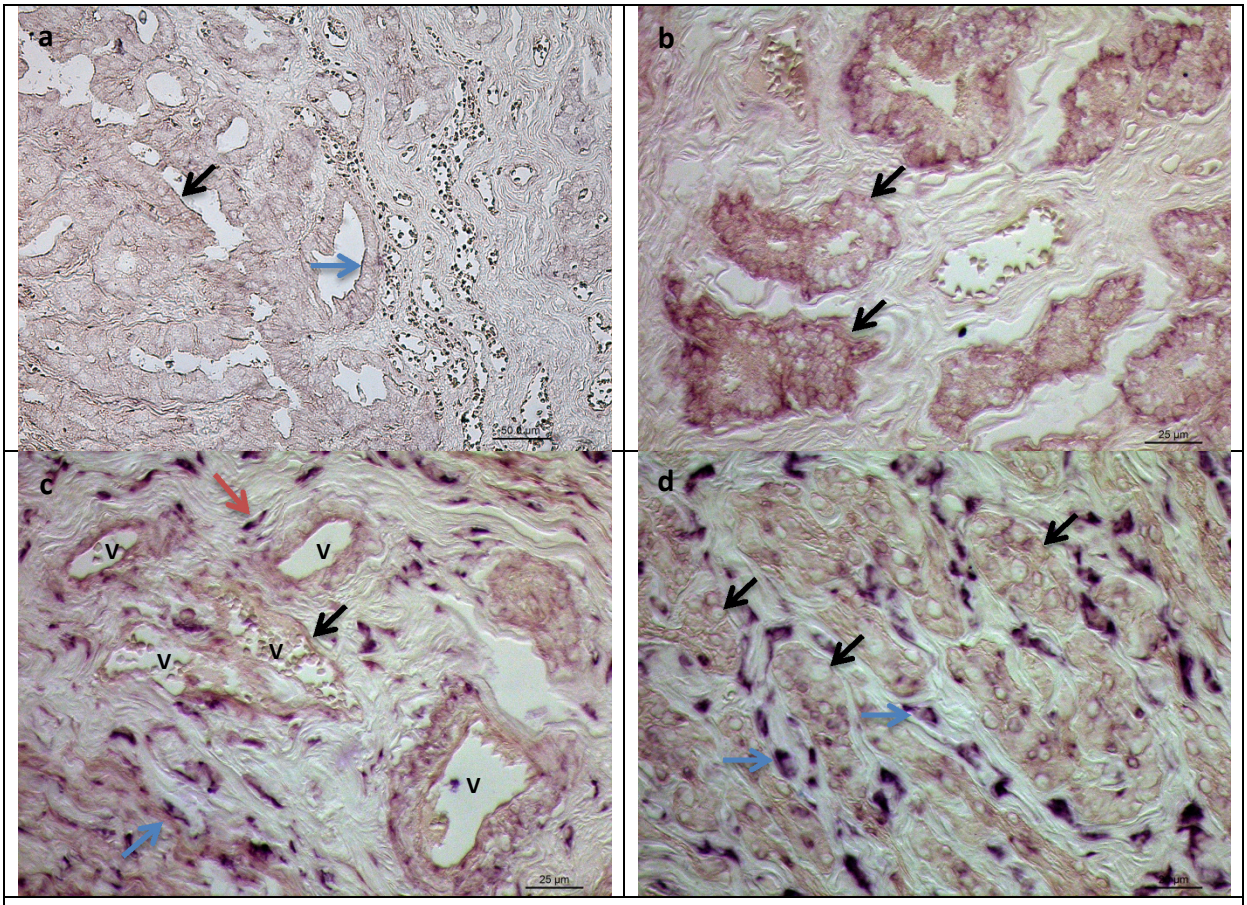


Figure 17:

a) Positive staining of the epithelial cells of the lamina epithelialis (black arrow) and superficial glands (blue arrow) (post-implantation group) (scale bar = 50 µm)
b) Deep glands (black arrows) with positive staining of the epithelial cells (post-implantation group) (scale bar = 25 µm)
c) Vessels (V) within the myometrial stratum vasculare with positively stained endothelial (black arrow) and smooth muscle cells (blue arrow) and surrounding fibroblasts (red arrow) showing positive signal (post-implantation group) (scale bar = 25 µm)
d) Stratum longitudinale of the myometrium with positive staining of the smooth muscle cells (black arrows) and stronger signals in the fibroblasts (blue arrows); in both cell types, the staining is more pronounced in the perinuclear region (post-implantation group) (scale bar = 25 µm)

7.2.2.2. Leptin receptor

In the inter-placental uterine sections of the post-implantation group, ObR mRNA had a similar distribution pattern as Ob mRNA. Positive signals were detected in the epithelial cells of the lamina epithelialis, the superficial and deep uterine glands. Furthermore, ObR mRNA was found in the myometrial and endometrial fibroblasts, and was especially pronounced close to the surface epithelium. Myometrial smooth muscle cells as well as endothelial and smooth muscle cells of vessels stained. In the fibroblasts and myometrial smooth muscle cells, the staining was more pronounced in the perinuclear region (Figure 18).

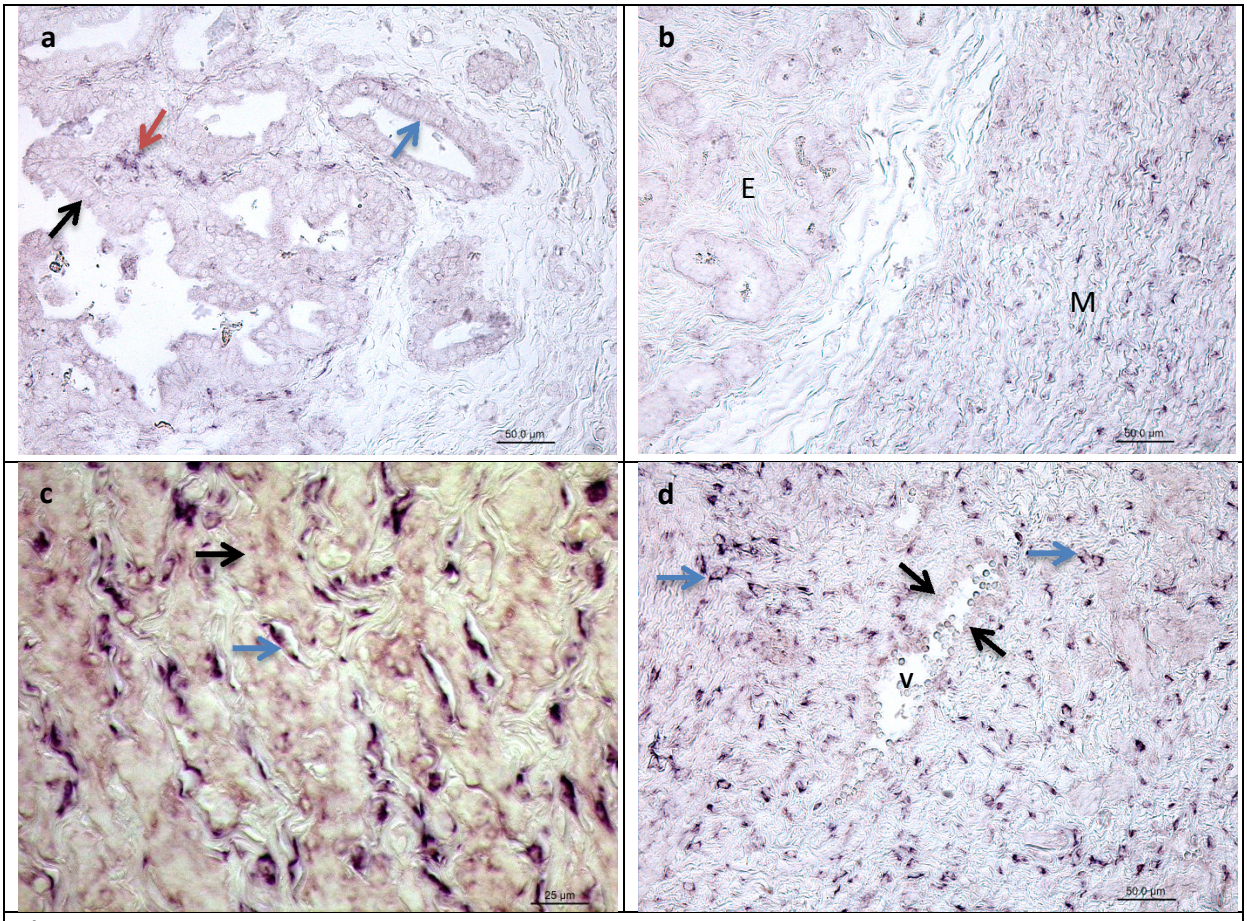


Figure 18:

a) Lamina epithelialis (black arrow) and superficial uterine glands (blue arrow) with positive staining for ObR in the epithelial cells; additional strong signal from endometrial stroma close to the surface epithelium (red arrow) (post-implantation group) (scale bar = 50 µm)

b) Endometrium (E) with deep glands and myometrium (M) with positive signals in the epithelial cells and smooth muscle cells/fibroblasts, respectively (post-implantation group) (scale bar = 50 µm)

c) Stratum longitudinale of the myometrium with positive signals in the smooth muscle cells (black arrow) and fibroblasts (blue arrow); in both cell types, the staining is more pronounced in the perinuclear region (post-implantation group) (scale bar = 25 µm)

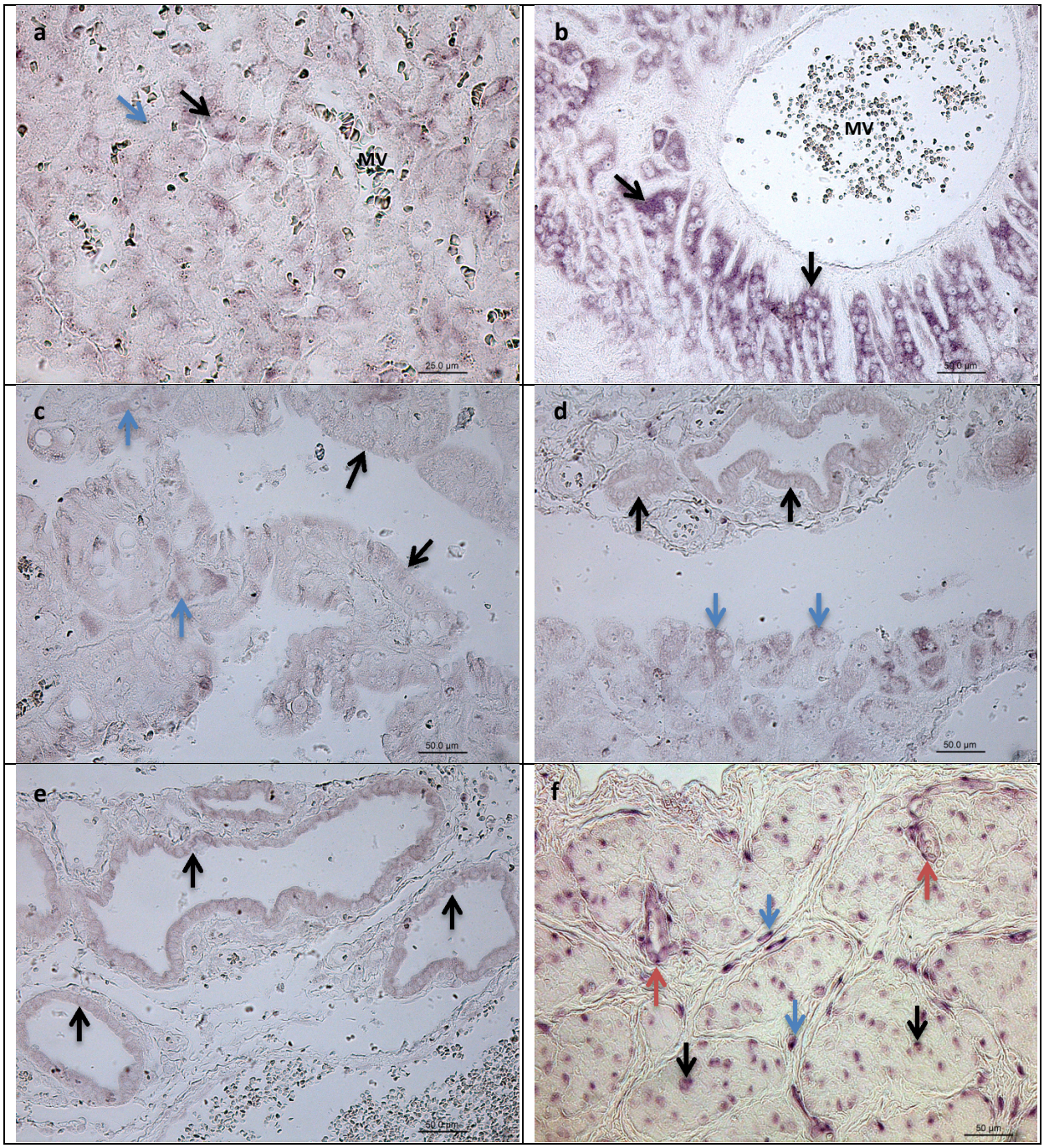
d) Blood vessel (V) within the stratum vasculare of the myometrium with positively stained endothelial cells (black arrows) surrounded by more intensely stained fibroblasts (blue arrows) (post-implantation group) (scale bar = 50 µm)

7.2.3. Utero-placental unit

7.2.3.1. Leptin

In the utero-placental unit of the prepartum luteolysis group, the distribution of ISH signals for Ob mRNA coincided with the protein signals detected by IHC. Ob mRNA was found in the placental labyrinth, where fetal trophoblasts stained more intensely than decidual cells. Invading trophoblasts around large maternal vessels at the base of the labyrinth at the feto-maternal contact zone showed strong positive signals. In the glandular chambers, both epithelial and stromal cells were weakly stained. Invading fetal trophoblasts in the cover layer of endometrial connective tissue near the deep glands showed positive signals. The deep uterine glands exhibited positive staining within the epithelial cells. Myometrial

smooth muscle cells and fibroblasts showed positive staining which was more pronounced in the perinuclear region. Endothelial and smooth muscle cells of vessels were sporadically stained as well (Figure 19).



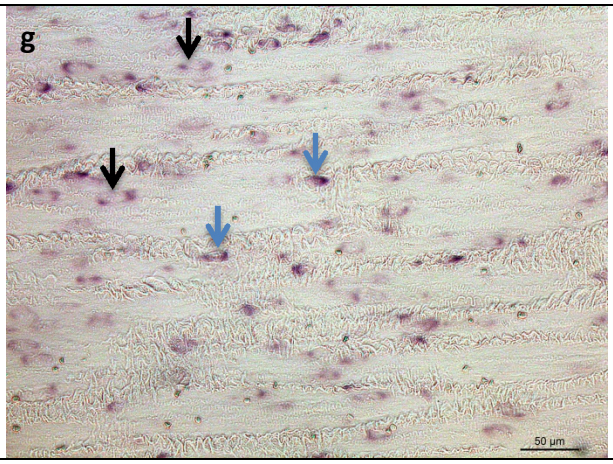


Figure 19:

a) Placental labyrinth: maternal vessels (MV) surrounded by weakly stained decidual cells (blue arrow) and more intensely stained fetal trophoblasts (black arrow) (prepartum luteolysis group) (scale bar = 25 µm)
b) Large maternal vessel (MV) at the base of the placental labyrinth surrounded by intensely positive invading fetal trophoblasts (black arrows) (prepartum luteolysis group) (scale bar = 50 µm)
c) Glandular chambers with weak staining in the epithelial (black arrows) and stromal cells (blue arrows) (prepartum luteolysis group) (scale bar = 50 µm)
d) Positive invading fetal trophoblasts (blue arrows) in the cover layer of endometrial connective tissue near the deep glands (black arrows) (prepartum luteolysis group) (scale bar = 50 µm)
e) Deep uterine glands with positive signals for Ob mRNA in the epithelial cells (black arrows) (prepartum luteolysis group) (scale bar = 50 µm)
f) Stratum longitudinale of the myometrium showing positive staining mainly in the perinuclear region of smooth muscle cells (black arrows) and fibroblasts (blue arrows) as well as in the endothelial cells of small vessels (red arrows) (prepartum luteolysis group) (scale bar = 50 µm)
g) Stratum circulare of the myometrium with signals for Ob mRNA mainly in the perinuclear region of the smooth muscle cells (black arrows) and fibroblasts (blue arrows) (prepartum luteolysis group) (scale bar = 50 µm)

7.2.3.2. Leptin receptor

ObR mRNA was detected in similar cell types as Ob mRNA. Positive signals were evident in the placental labyrinth, where fetal trophoblasts stained more intensely than decidual cells. Invading trophoblasts around large maternal vessels at the base of the labyrinth showed strong positive signals. There was ObR mRNA detectable in trophoblasts migrating from the placental labyrinth towards the glandular chambers. In the glandular chambers epithelial cells had sporadic weak signals. The deep uterine glands showed positive staining within the epithelial cells. Myometrial smooth muscle cells and fibroblasts stained positive which was more pronounced in the perinuclear region similarly to Ob. Endothelial and smooth muscle cells of vessels in the stratum vasculare of the myometrium also showed positive signals (Figure 20).

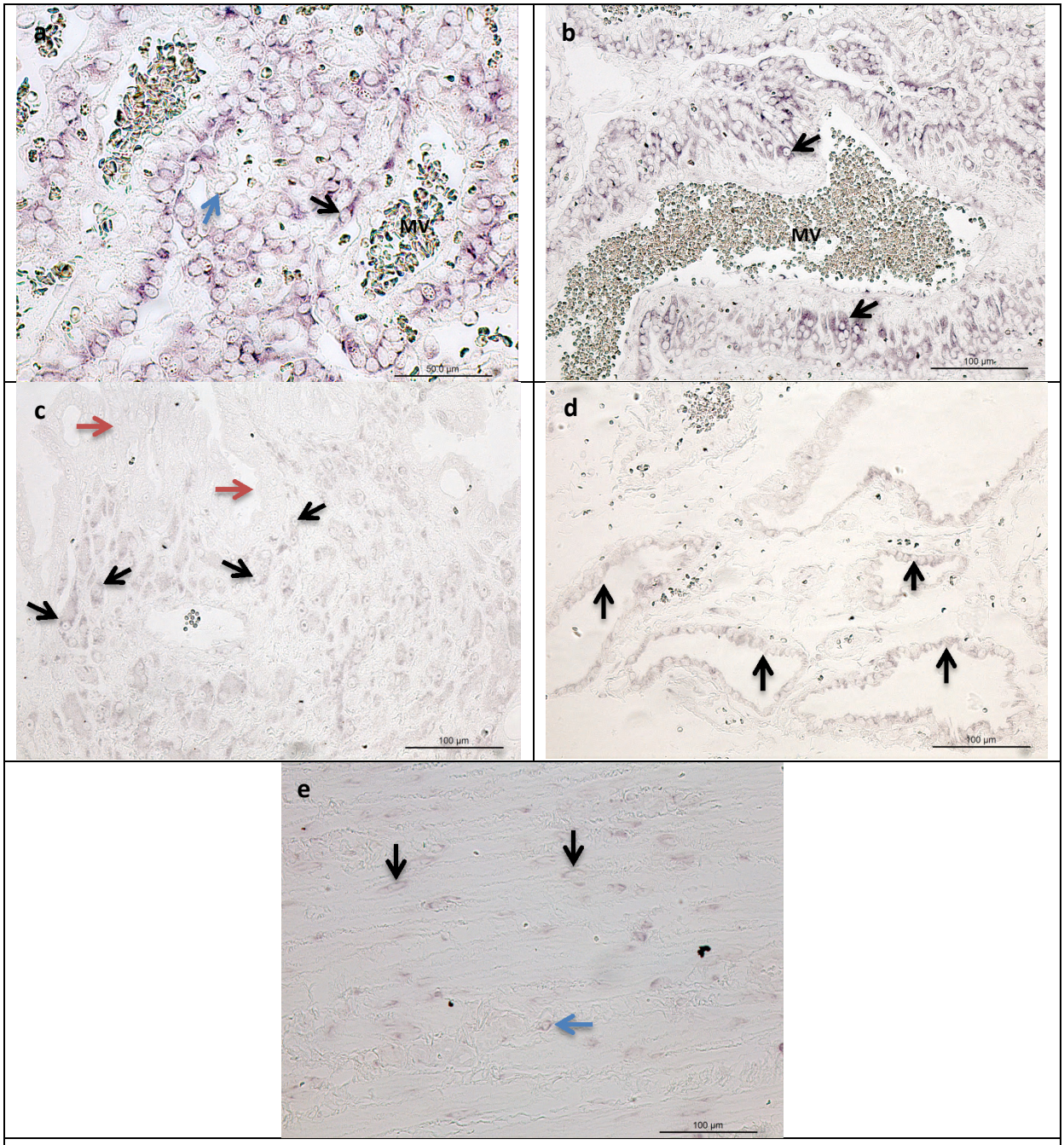


Figure 20:

a) Placental labyrinth: maternal vessels (MV) surrounded by weakly stained decidual cells (blue arrow) and more intensly stained fetal trophoblasts (black arrow) (prepartum luteolysis group) (scale bar = 50 µm)

b) Large maternal vessel (MV) at the base of the placental labyrinth surrounded by intensely stained invading fetal trophoblasts (black arrows) (prepartum luteolysis group) (scale bar = 100 µm)

c) Positively stained fetal trophoblasts (black arrows) migrating from the placental labyrinth towards the glandular chambers; no or very weak signals detectable in the epithelial cells of glandular chambers (red arrows) (prepartum luteolysis group) (scale bar = 100 µm)

d) Deep uterine glands with positive signal for ObR mRNA in the epithelial cells (black arrows) (prepartum luteolysis group) (scale bar = 100 µm)

e) Stratum circulare of the myometrium: positive signal for ObR mRNA mainly in the perinuclear region of the smooth muscle cells (black arrows) and fibroblasts (blue arrow) (prepartum luteolysis group) (scale bar = 100 µm)

7.2.4. Negative controls

Sense hybridization probes were used as negative controls. There were no signals detected in any of the negative control slides. Some representative slides are shown in Figure 21.

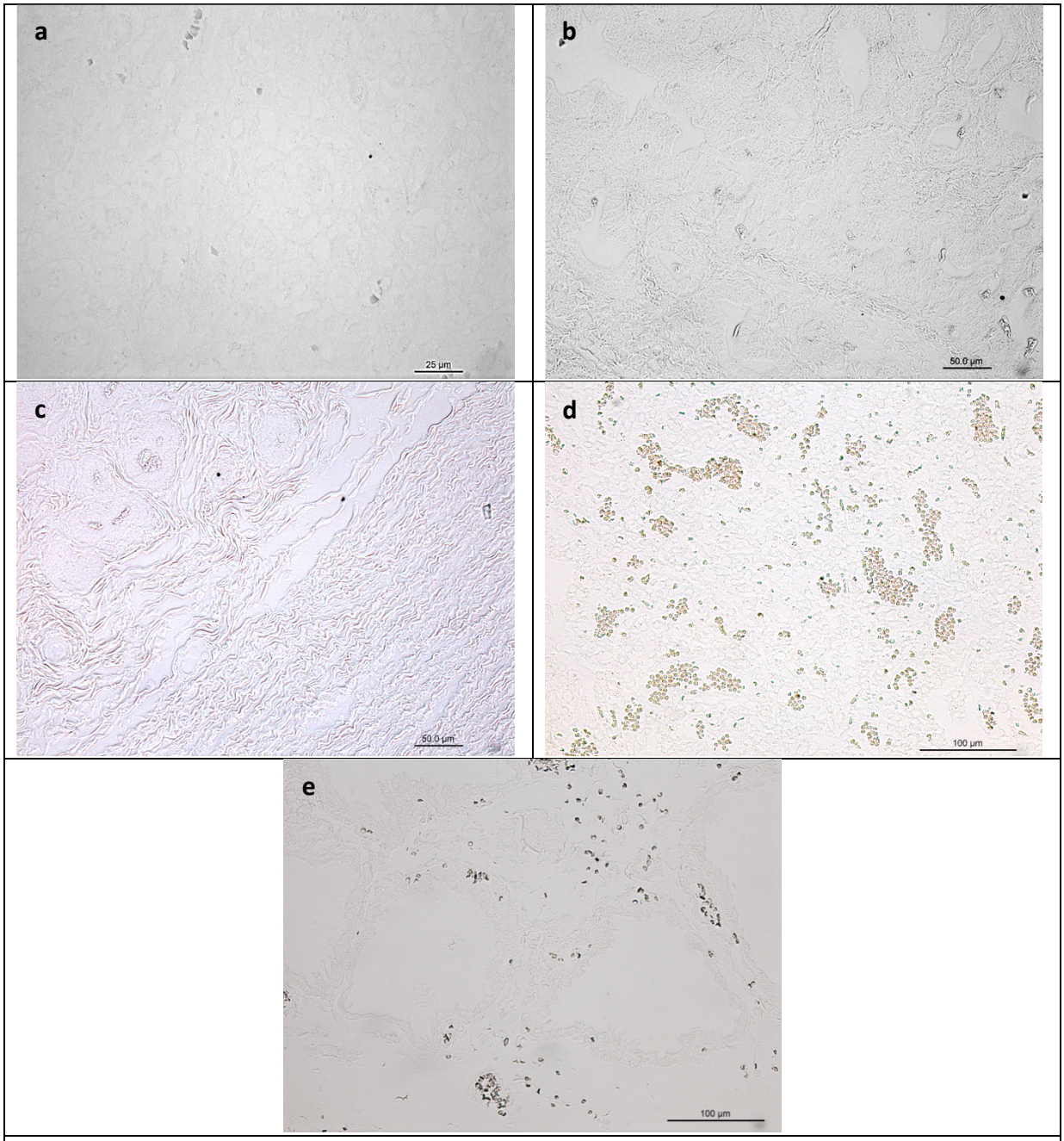


Figure 21:

a) CL post-implantation, Ob (scale bar = 25 µm)
b) Inter-placental uterine section, post-implantation, Ob: Lamina epithelialis and superficial glands (scale bar = 50 µm)
c) Inter-placental uterine section, post-implantation, ObR: deep uterine glands on the left and myometrium on the right side (scale bar = 50 µm)
d) Utero-placental unit, prepartum luteolysis, Ob: placental labyrinth (scale bar = 100 µm)
e) Utero-placental unit, prepartum luteolysis, ObR: deep uterine glands (scale bar = 100 µm)

7.3. Semi-quantitative Real Time (TaqMan) PCR

7.3.1. Pre-implantation uterus and inter-placental uterine sections

7.3.1.1. Leptin

There was no significant difference in the expression of Ob mRNA between pre-implantation, post-implantation and mid-gestation (P = 0.118), although numerically a gradual decrease was noted from early to mid-gestation (Figure 22a).

7.3.1.2. Leptin receptor

ObR gene expression was significantly higher in the uterus and inter-placental uterine sections during pre- and post-implantation compared to mid-gestation (P = 0.016 and P = 0.019, respectively) (Figure 22b).

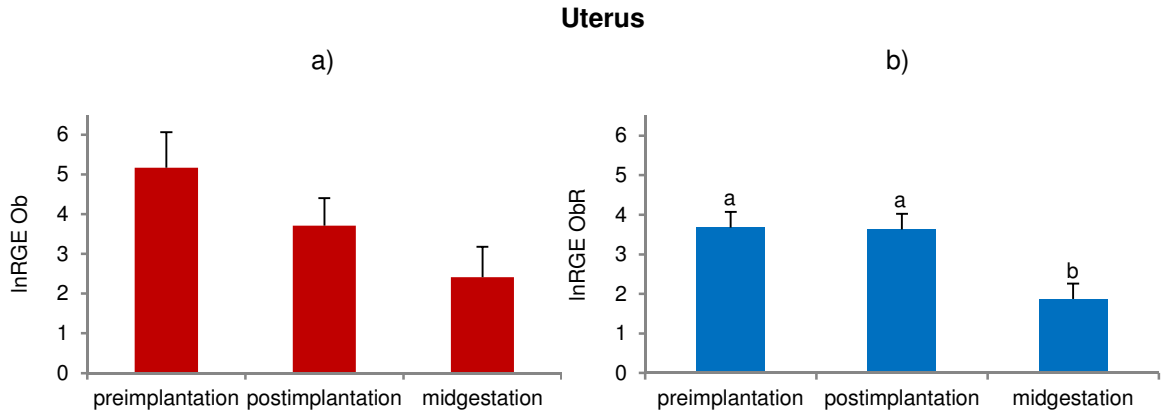


Figure 22: (a) Relative leptin gene expression in the pre-implantation uterus (n=3) and at inter-placental uterine sections at post-implantation (n=5) and mid-gestation (n=4). (b) Relative leptin receptor gene expression in the pre-implantation uterus (n=5) and at inter-placental uterine sections at post-implantation (n=5) and mid-gestation (n=5). Only valid data was analysed and is shown here. Different superscripts denote significant differences between stages. Bars represent the mean and whiskers the SE. lnRGE: relative gene expression after log-transformation of observed data.

7.3.2. Utero-placental unit

7.3.2.1. Leptin

Leptin mRNA expression was significantly higher at post-implantation and at prepartum luteolysis compared to mid-gestation (P = 0.043 and P = 0.014, respectively) (Figure 23a).

7.3.2.2. Leptin receptor

ObR mRNA levels were significantly up-regulated during prepartum luteolysis compared to post-implantation (P < 0.001) and mid-gestation (P < 0.001). ObR expression in the post-implantation group was similar to mid-gestation (P = 0.211) (Figure 23b).

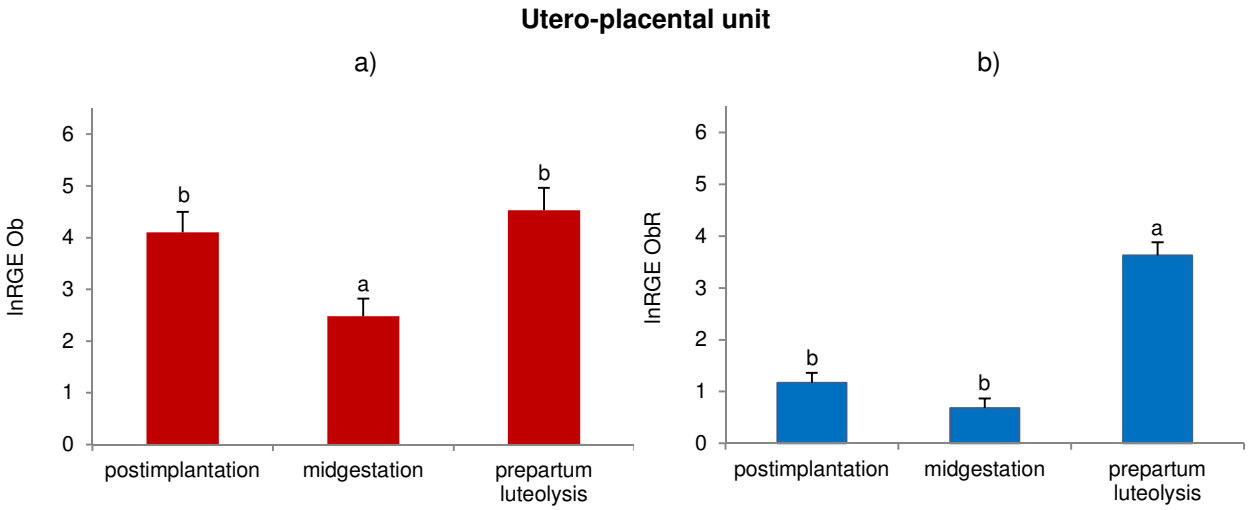


Figure 23: (a) Relative leptin gene expression in the utero-placental unit at post-implantation (n=3), mid-gestation (n=5) and prepartum luteolysis (n=3). (b) Relative leptin receptor gene expression in the utero-placental unit at post-implantation (n=5), mid-gestation (n=5) and prepartum luteolysis (n=3). Only valid data was analysed and is shown here. Different superscripts denote significant differences between stages. Bars represent the mean and whiskers the SE. lnRGE: relative gene expression after log-transformation of observed data.

7.3.3. Comparison of early pregnant and non-pregnant diestrous uteri

7.3.3.1. Leptin

In the E+ group, Ob gene expression was detected in the uterus of 4 out of 8 bitches, while it was not found in any of the non-pregnant animals that were matched for reproductive cycle stage but had no embryos on uterine lavage post-ovariohysterectomy (n=6). The figure below shows the mean and SE of observed data in the 4 pregnant dogs. No statistical analysis was carried out (Figure 24).

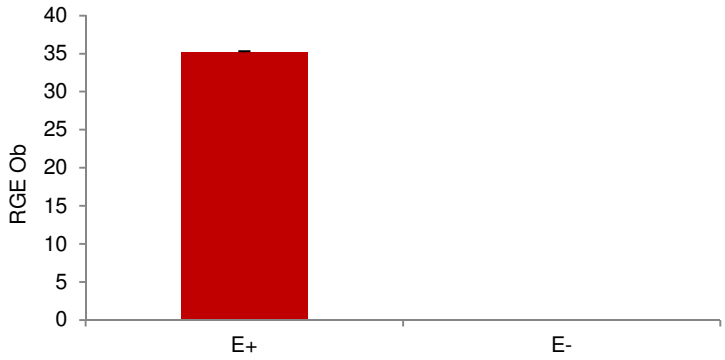


Figure 24: Relative leptin gene expression in the uterus of pregnant bitches in the pre-implantation period (E+). The bar represents the mean and whisker the SE of the 4 pregnant dogs where Ob expression was detected. No Ob mRNA expression was found in the non-pregnant group (E-). RGE: relative gene expression (observed data)

7.3.3.2. Leptin receptor

ObR mRNA was detected in the uterus of all animals, however, there was no significant difference in the gene expression levels between E+ and E- bitches (P = 0.363) (Figure 25).

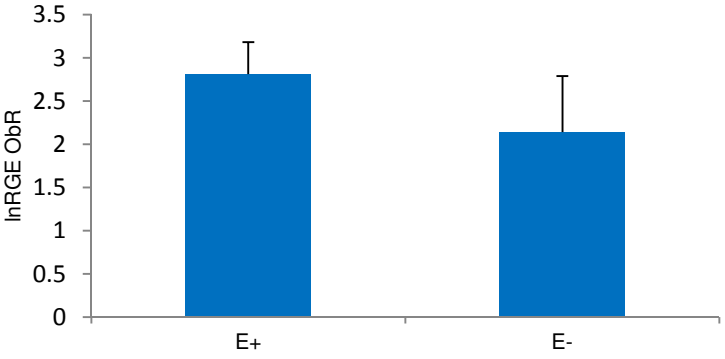


Figure 25: Relative leptin receptor gene expression in the uterus of non-pregnant dogs in early diestrus (E-) and in pregnant bitches at the pre-implantation period (E+). Bars represent the mean and whiskers the SE. lnRGE: relative gene expression after log-transformation of observed data

8. Discussion

The present study shows, for the first time, Ob and ObR mRNA and protein expression during all stages of pregnancy in various cell types of the canine CL, uterus and placenta.

In the corpus luteum, we detected immunoreactivity for Ob and ObR primarily in the luteal cells during all stages of pregnancy. These findings support the results previously published by our group showing the presence of Ob and ObR mRNA in the canine CL in the course of pregnancy by semi-quantitative real-time (TaqMan) PCR [7]. Ob staining was evenly and homogeneously distributed in the cytoplasm of luteal cells, whereas there was a more heterogeneous and granular staining observed for ObR. This difference might be due to a different expression pattern of the proteins. Canine ObR expression may be accentuated in the area of production, i.e. the endoplasmatic reticulum. However in other species (e.g. buffalo and Japanese black bear), ObR staining was homogenously distributed within the cytoplasm of luteal cells [106, 146].

ObR was detected during all stages of pregnancy in both luteal and endothelial cells, whereas endothelial Ob protein was only detected in the later stages of pregnancy (in the mid-gestation and prepartum luteolysis groups). However, ISH revealed Ob mRNA expression in the endothelial cells already at post-implantation, and unfortunately other groups were not tested. This may be explained, at least in part, with differences in the processes of transcription and translation, or lower levels of the endothelial Ob protein compared to later pregnancy stages. However, in general, the presence of Ob and ObR in endothelial cells of the CL may imply a regulatory role in luteal angiogenesis and blood flow in the dog. In corpora lutea during different stages of the estrous cycle of non-pregnant buffalos Ob and ObR protein were detected by IHC in the cytoplasm of luteal cells, but only ObR was found in endothelial cells [106]. The fact that Ob was not found in endothelial cells may be attributable to species-specificity.

No ObR protein expression in the CL of normal non-pregnant female dogs was found in a study of 2006 [6], whereas we detected ObR protein in the CL during all stages of gestation. Reasons for this discrepancy could be the difference in ObR protein expression levels between pregnant and non-pregnant dogs. However, as previously described by our group, at the mRNA level, ObR expression was found in the CL of non-pregnant bitches during diestrus, although ObR protein was not examined by IHC, and gene expression levels were not compared to that of pregnant dogs [7]. Therefore, these contradictory findings may be explained by a different IHC procedure or by another antibody used.

In our study subjectively stronger immunostaining for Ob was detected in the luteal cells during post-implantation compared to the other pregnancy phases however we did deliberately not judge the slides in a quantitative way. This up-regulation of Ob is coincident with generally high blood levels of progesterone [23]. That leads to a possible explanation for our IHC findings, as Siawrys et al. showed that progesterone increased Ob secretion by porcine luteal cells *in vitro* [145]. Thus, the up-regulation of luteal Ob mRNA might be

triggered by high serum progesterone levels. On the other hand, it may indicate a luteal steroidogenic function of Ob, like it was already discussed by Kumar et al. [106], where the expression of key steroidogenic factors (e.g. steroidogenic acute regulatory protein (StAR)) correlated with Ob and ObR activity in luteal cells. Also Ruiz-Cortés et al. [154] report on a stimulatory effect of Ob on progesterone production by porcine non-luteinized granulosa cells. Although the presenting authors cannot answer this chicken-and-egg debate, they assume based on their IHC results a positive correlation of Ob and progesterone, which is in contrast to the study on human granulosa cells stating an inhibitory effect of Ob on steroidogenesis [152].

Semi-quantitative RT-PCR of the pre-implantation uterus and inter-placental uterine sections revealed a down-regulation of ObR mRNA in the course of pregnancy with the lowest expression in the mid-gestation group and significantly higher expression during the earlier stages, while Ob expression did only decrease numerically but not statistically significant during the course of pregnancy. This might support the findings of Malik et al. [191] stating that Ob is only necessary for a successful murine pregnancy during the time of implantation. In their study, no pregnancy was resulting in Ob-deficient mice when exogenous Ob administration was stopped 0.5 or 3.5 days post coitus, whereas Ob-withdrawing at later time points did not affect pregnancy. In humans, Dos Santos et al. found significantly lower endometrial Ob expression in women with implantation failure compared to fertile women [188].

Our results do not exclude Ob's importance during the rest of the pregnancy, but do also point out Ob's importance in uterine receptivity and in the preparation for implantation. This is further supported by the results of experiment 2, where Ob expression was found in the pre-implantation uteri (4 out of 8 of E+) and in none of the non-pregnant bitches. By IHC, both Ob and ObR protein and mRNA were localized in the epithelial cells of the lamina epithelialis, the superficial and deep glands of pre-implantation uteri, which is in close contact with the developing embryo. It is possible that Ob is only expressed in the uterus when an embryo is there, which is then able to activate ObR to signal its presence. To the author's opinion, these findings indicate a possible involvement of Ob/ObR in the autocrine/paracrine regulation of uterine receptivity, embryo-maternal cross-talk and embryonic development also in the dog, as it was suggested in other species [52, 189, 190].

In experiment 2, Ob mRNA in the uterus of the E+ group was detected in 4 animals, but was below our detection limit in the remaining 4 dogs. In experiment 1, Ob was not detected in 2 out of 5 dogs in the pre-implantation stage. Possible explanations for this might be individual differences, timing of tissue collection (as the range was between 8 and 12 days after mating) even though it was tightly timed, or differences in harvesting practices that might have led to degradation of the mRNA in some tissue samples. In another study Ob gene expression was investigated in the early diestrus uterus of non-pregnant dogs as well as in the pre-implantation uterus and embryo in bitches, but no Ob mRNA was found in any of these samples [247]. However, the authors used a qualitative PCR, so the sensitivity of

detection of their method may be lower than the semi-quantitative real-time PCR employed here. By IHC, we detected Ob protein in all of the pre-implantation uteri of experiment 1, albeit this protein may be circulating and originate from somewhere else e.g. the adipose tissue.

In the utero-placental units, both Ob and ObR mRNA expression was significantly up-regulated towards the end of pregnancy compared to mid-gestation. This may, at least in part, be due to increased placental (and probably also myometrial) tissue mass. However, Ob mRNA levels were significantly higher also in post-implantation compared to mid-gestation, which could not be explained by a larger placenta but imply a physiological up-regulation of Ob gene expression at this earlier stage. Similarly, this might also be true at the prepartum luteolysis phase, although the reason for the increased placental Ob and ObR gene expression is unclear. An immune-modulatory function in preparation for parturition has to be taken into account considering that Ob is structurally similar to the type I cytokine superfamily [46], cytokines play an important role in the regulation of delivery [248], and Ob increased significantly the release of cytokines including PGF2α from human placenta [249]. Furthermore, an involvement in the process of placental separation (maybe via activation/enhanced secretion of MMPs [5], which were shown to be expressed in late gestation canine placentas [250]), or a role in fetal pulmonary development [183] could be considered. In contrast to our study, in baboon pregnancy placental Ob mRNA was negatively correlated with gestational age [160]. However, these samples were not from prepartum luteolysis or from parturition, and the placental tissue was separated from uterine tissue. In dogs studies are needed on separated uterine and placental tissue to ascertain to which tissue Ob and ObR gene expression are attributable. Additionally, the different isoforms of the receptors should be distinguished in further studies as they might respond in different ways to the presence of Ob (e.g. buffering effect of the soluble isoform) and thus modulate the feto-maternal communication differently.

Both in IHC and ISH, trophoblasts within the placental labyrinth stained stronger for Ob and ObR mRNA and protein than decidual cells. Hoggard et al. detected Ob and ObR mRNA and protein in murine placenta, but specific cellular localization was not described [69]. Bodner et al. [197] investigated the expression and localization of ObR in human term placenta. They found positive signals in the syncytiotrophoblasts using IHC and ISH, but decidual cells did not stain in either method. Similar results were obtained by Challier et al. [251]. Guibourdenche and co-workers detected Ob protein in isolated human cyto-/syncytiotrophoblasts by immunocytochemical staining [252]. Cervero and her colleagues report immunoreactivity for ObR in human trophoblasts and decidual cells in 8-week-old human implantation sites [2]. Data on the localization of Ob and ObR expression in the placenta of domestic animals is scarce. Dall' Aglio and her group [253] studied the presence and distribution of Ob and ObR in the cat placenta at approximately 55-60 days of pregnancy by means of IHC. They localized Ob and ObR protein in the perinuclear region of both decidual cells and syncytiotrophoblasts, which is in accordance with our results, even though they did not detect a difference in staining intensity between decidual and trophoblast cells.

In contrast to our findings, no other uterine structure outside the placental girdle stained positive for Ob and ObR. Cumulatively, the detection of Ob and ObR in both trophoblasts and weakly in decidual cells indicates that the placenta is not only a site of Ob production but also a target for its effects, and Ob might act in a paracrine/autocrine manner to modulate placental function in the dog. It may stimulate placental and fetal growth by promoting placental angiogenesis [98] (Ob and ObR mRNA and protein were also detected in endothelial cells of placental vessels) and nutrient transport similarly to humans [200] and rodents [201]. Furthermore, it may promote trophoblast invasion through increasing the secretion and/or activity of matrix metalloproteinases [5, 198, 254] and thus implantation and placenta formation.

The detection of both Ob and ObR protein and mRNA in myometrial smooth muscle cells might imply a role for Ob in myometrial function in an autocrine/paracrine and/or endocrine manner. In human medicine [206, 207], high Ob levels were shown to inhibit uterine contractions. German already listed dystocia as an obesity-associated disease in dogs but attributed it to excess fat tissue in and around the birth canal [217] without mentioning a possible inhibitory effect of Ob directly on the myometrium. In our study, inter-placental uterine sections were not available in the prepartum luteolysis group, however, in the utero-placental unit, both Ob and ObR mRNA were significantly up-regulated during prepartum luteolysis compared to midgestation. Because myometrial tissue expression was not examined separately from that of the endometrium and placenta, we cannot draw any conclusions on the changes in local myometrial Ob and ObR mRNA levels. Although both proteins were found by IHC, quantification was not intended. Further studies using e.g. laser capture microdissection would be needed to examine in more details Ob's role in myometrial function during canine pregnancy and parturition, and to assess obesity as a risk factor for uterine inertia in dogs. Besides its possible tocolytic effect, Ob might act as a growth factor on the myometrium (it was also found in myometrial endothelial cells) by enhancing myometrial angiogenesis [98].

The detection of both Ob and ObR protein and mRNA in blood vessel endothelium of several tissue compartments studied e.g. the CL, placenta, endometrium and myometrium suggests a possible role for Ob in angiogenesis also in dogs as reported for other species [98, 100, 101, 103]. Quantifying endothelial Ob and ObR expression in canine reproductive tissues in the course of pregnancy after e.g. laser capture microdissection would perhaps allow us to draw more precise conclusions about Ob's involvement in the angiogenic aspects in pregnancy.

Fibroblasts stained positive for Ob and ObR in IHC in endometrial, myometrial and placental tissue, but not in the CL. Yoon et al. found higher ObR expression in stromal cells than in uterine luminal epithelium during the implantation window in mice [193]. Whether this finding is of importance has yet to be determined. A possible regulatory role of stromal Ob/ObR in an endocrine and/or paracrine/autocrine manner (e.g. tissue growth, embryo-maternal communication) can only be speculated.

In the literature review of this thesis, a lot of studies from humans and rodents were mentioned. The reason for this is the large amount of data gathered about Ob's role in reproduction in these species. Nevertheless, the canine reproductive cycle and pregnancy is different from the human or murine one and findings obtained in one species cannot be extrapolated directly to another. This study is the first to address leptin signalling in the uterus and placenta during canine gestation and at prepartum luteolysis.

We conclude that Ob and its receptor may be involved in the establishment and maintenance of canine pregnancy in a paracrine/autocrine manner. Our results suggest a regulatory role for the Ob-ObR system in luteal function, endometrial receptivity, implantation, trophoblast invasion, endothelial function and angiogenesis in reproductive tissues.

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12. Appendices

12.1. Buffers

12.1.1. Citrate buffer

Stock solution A (0.1 M citric acid):

$C_6H_8O_7$ x H_2O	21 g
Sterile double-distilled water	Ad 1000 ml

Stock solution B (0.1 M sodium citrate):

$C_6H_5O_7Na_3$ x 2 H_2O	28.41 g
Sterile double-distilled water	Ad 1000 ml

Working solution:

Stock solution A	9 ml
Stock solution B	41 ml
Sterile double-distilled water	Ad 500 ml

12.1.2. Immunohistochemistry-buffer (IHC buffer)

Stock solution (IHC buffer 10x, pH 7.2-7.4):

Na_2HPO_4	12 g
KH_2PO_4	2 g
KCl	2 g
NaCl	80 g
Sterile double-distilled water	Ad 1000 ml

Working solution (IHC buffer 1x/ 0,3% Triton X-100, pH 7,2-7,4):

IHC buffer 10x	100 ml
Sterile double-distilled water	Ad 1000 ml
Triton X-100	3 ml (after adjusting the pH to 7,2-7,4)

12.1.3. Phosphate buffered saline (PBS)

Stock solution (PBS 10x)

NaCl	80 g
KCl	2 g

Na_2HPO_4	14.4 g
NaH_2PO_4	2.4 g
Sterile double-distilled water	Ad 1000 ml

Working solution (PBS 1x)

PBS 10x	100 ml
Sterile double-distilled water	Ad 1000 ml

12.1.4. In situ hybridization buffer 1 (ISH buffer 1) pH 7.5

Tris HCl	12.7 g
Tris Base	2.36 g
NaCl	8.76 g
Sterile double-distilled water	Ad 1000 ml

12.1.5. In situ hybridization buffer 2 (ISH buffer 2) pH 9.6

Tris HCl	0.76 g
Tris Base	5.47 g
NaCl	2.92 g
$MgCl_2$	2.38 g
Sterile double-distilled water	Ad 500 ml

12.1.6. Phosphate buffered saline with Tween 20 (PBST)

PBS 10x	100 ml
Sterile double-distilled water	Ad 1000 ml
Tween 20	2.5 ml

12.1.7. 20x Saline-sodium citrate buffer (20x SSC buffer)

$C_6H_5O_7Na_3 \times 2\ H_2O$	88.23 g
NaCl	175.29 g
Sterile double-distilled water	Ad 1000 ml